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URINARY PORPHYRINS IN DISEASE

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The structural chemistry of the blood and bile pigments has
been developed by Hans Fischer and his school (1), but the phys-
iological conditions which lead to the increased production of these
pigments, especially of the porphyrins, are still uncertain. Al-
though it is well known that in many diseases, notably those char-
acterized by fever or liver injury (2), an increased excretion of
porphyrins in the urine occurs, there are few data as to the kind
of porphyrin excreted. From the study of the urinary porphyrins
of congenital porphyrinuria and the scattered results of the few
non-idiopathic porphyria which have been investigated, it is
evident that only porphyrins derived from etioporphyrin I or III
are to be expected in the urine (1, 3, 4). The question remains,
however, as to what porphyrins are excreted in various diseases
and what significance may be attached to their excretion. To
answer the first of these questions the porphyrins from the urine
of a variety of cases were isolated and characterized. Predomi-
nant among the diseases studied were those in which malfunction
of the liver was the outstanding phenomenon.

Methods

The methods used were modifications of the methods developed
by Fischer (5, 6) and Garrod (7). The porphyrins were separated
from the urine by precipitation and absorption, purified, and
characterized spectroscopically, and finally identified by con-
version to the methyl esters. The scheme is summarized in
Table I and described in detail below.[1]

[1] Provision is made in the scheme for the detection of proto- and deutero-
porphyrins, although these porphyrins were not found in the urines exam-
ined. The scheme is thus generally valid for stool as well as urine.

1

A. Precipitation of Porphyrins—In diffuse light fresh, unfiltered urine is treated with one-twentieth of its volume of 40 per cent sodium hydroxide and to each 1500 cc. of urine 5 cc. of saturated calcium chloride solution are added with vigorous stirring. The voluminous precipitate of phosphates which results carries down nearly quantitatively all the porphyrins of the urine. As the precipitate settles it is at first repeatedly stirred or the mixture shaken. When settling is complete, the supernatant liquid is decanted and the precipitate collected by filtration or, better, by

TABLE I

Scheme for Separation of Urinary Porphyrins

Crude porphyrins dissolved in concentrated hydrochloric acid; the solution neutralized with sodium acetate-acetic acid and extracted with ether

Ether-insoluble porphyrins, *uroporphyrins* (B, F)	Ether solution shaken with 5% hydrochloric acid and the acid solution then extracted with chloroform		
	Chloroform-soluble porphyrins, *protoporphyrins* (C, D)	Reduce acid solution to 0.2% HCl and reextract with chloroform	
		Chloroform solution, *deuteroporphyrins* (C, D)	Neutralize acid solution with sodium acetate-acetic acid and extract with ether. Ether solution, *coproporphyrins* (E)

The letters in parentheses refer to sections of the text.

centrifugation. Since ammonia renders the removal less quantitative, care should be taken that ammoniacal fermentation of the urine has not occurred.

B. Separation of Ether-Soluble and Ether-Insoluble Porphyrins— The phosphate precipitate is dissolved in the least possible volume of concentrated (12 N) hydrochloric acid, the solution neutralized to Congo red by the addition of solid sodium acetate, and then one-third of its volume of glacial acetic acid added. From this weakly acid solution the ether-soluble porphyrins are removed by triple extraction with portions of ether equal in volume to that of

the solution. Should an emulsion be formed during extraction, separation of the layers may be brought about by the addition of glacial acetic acid or a few cc. of ethyl alcohol. The combined ether extracts are then shaken two or three times with one-third of their volume of distilled water and the ether solution filtered. The wash water and original solution are combined and utilized for the isolation of the ether-insoluble porphyrins (Section F).

C. Separation of Ether and Chloroform-Soluble Porphyrins— The ether solution, which contains ether- and chloroform-soluble porphyrins, is repeatedly extracted with small amounts of 5 per cent hydrochloric acid. Occasionally porphyrins are retained in the ether due to the presence of colloids and in such cases it is necessary to extract with 10 or 25 per cent hydrochloric acid, the solution resulting being then diluted to 5 per cent. The 5 per cent hydrochloric acid solution is neutralized by the addition of solid sodium acetate-acetic acid and the porphyrins extracted with ether. This procedure of driving the porphyrins back and forth between ether and hydrochloric acid is repeated several times, completeness of extraction being controlled by spectroscopic examination or by the absence of fluorescence in ultra-violet light. Finally a 5 per cent hydrochloric acid solution is extracted with chloroform to remove any blue bile pigments (biliviolin) and protoporphyrins.

The 5 per cent hydrochloric acid solution of the porphyrins is again neutralized with sodium acetate and the porphyrins extracted with ether.[2] After being reextracted with 0.4 per cent hydrochloric acid, the acid solution is diluted with an equal volume of water and the resulting 0.2 per cent solution repeatedly extracted with small amounts of chloroform to remove any deuteroporphyrins.

During the extraction of the 0.2 per cent hydrochloric acid solution with chloroform, coproporphyrin may precipitate at the interface between the acid and chloroform. In this case filtration and subsequent solution of the precipitate in hydrochloric acid is the most convenient method of avoiding loss.

D. Chloroform-Soluble Porphyrins—The chloroform solutions are examined separately with a spectroscope for the presence of

[2] Dilution of the 5 per cent hydrochloric acid solution would result in excessively large volumes.

porphyrins. If present, each solution is filtered through double filter paper, the chloroform evaporated *in vacuo*, and the porphyrins purified as described above.

E. Ether-Soluble, Chloroform-Insoluble Porphyrins (Coproporphyrins)—After extraction of the 0.2 per cent hydrochloric acid solution with chloroform, it is neutralized with sodium acetate, reextracted with ether, and the porphyrins concentrated into a small volume of 10 per cent hydrochloric acid by extraction of the ether solution. The hydrochloric acid solution is neutralized to Congo red by addition of sodium acetate and a few drops of 30 per cent acetic acid are added. Within 48 hours the coproporphyrins are quantitatively precipitated. They are removed by filtering with suction, or by centrifuging, and then esterified. Where only a small quantity of porphyrin is present, as is true in the majority of cases, it is more advantageous to evaporate the purified ether solution and esterify.

F. Ether-Insoluble Porphyrins (Uroporphyrins)—The original aqueous solution (see Section B), together with the wash waters, is allowed to stand for 48 to 72 hours. The resulting precipitate is removed by filtering or centrifuging and combined with the precipitate remaining from the filtration of the ether solution (Section B). The combined precipitates are dissolved in 10 per cent hydrochloric acid, filtered free from insoluble material, and examined spectroscopically for porphyrins. If present, the solution is tested once more for the presence of ether-soluble porphyrins by neutralizing to Congo red with sodium acetate-acetic acid and spectroscopic examination of an ether extract. Any ether-soluble porphyrins are removed by extraction and the ether extract combined with the material utilized in the separation discussed in Section C. When ether-soluble porphyrins are absent, a few cc. of glacial acetic acid are added to the aqueous solution of the uroporphyrins and the mixture allowed to stand for 48 hours, when quantitative precipitation occurs. The separated porphyrins may be further purified by repetition of the procedure.

Where only uroporphyrin is present in the urine, it may be precipitated quantitatively with the urates by the addition of 50 cc. of concentrated glacial acetic acid to each liter of urine. In this case the precipitate should be filtered after 8 days of standing and purified as described above.

Esterification of Porphyrins—The ether or chloroform solution of the porphyrin is run into a 25 to 50 cc. distillation flask with simultaneous flash distillation of the solvent. To the residue 15 to 30 cc. of methyl alcohol, saturated with hydrogen chloride, are added and the mixture stoppered. After standing for 24 hours at room temperature, esterification is generally complete and the excess methylating mixture may be removed by distillation under a vacuum. The ester is taken up in a small amount of chloroform and shaken with 2 per cent sodium carbonate until the chloroform solution shows the "alkaline" porphyrin spectrum. Unesterified porphyrins in the soda solution and insoluble residues should be subjected to a second esterification.

The chloroform solution is repeatedly washed with small amounts of distilled water until no flocculent particles are left, filtered through a double folded filter, and then evaporated to dryness on the water bath. The ester is taken up in fresh chloroform, transferred to a 15 cc. conical centrifuge tube, and the volume reduced to 2 cc. by warming. An equal volume of methyl alcohol is added and the solution concentrated to one-half its volume. The porphyrin esters precipitate at once, or after standing, and are separated from the mother liquor by centrifuging. Additional amounts of ester may be obtained from the mother liquor.

Final purification is effected by recrystallizing four to five times from methyl alcohol-chloroform, the last crystallization being carried out on small watch-glasses. It is often advantageous previous to the chloroform extraction to wash the impure ester by decantation with hot petroleum ether even though some loss results thereby.

Identification of Porphyrins—The porphyrins were identified by means of the crystal form, the acid number, and the absorption spectrum. In general, spectroscopic comparison was made with known specimens of porphyrins by projecting the spectrum of the known porphyrins over that of the unknown. Final identification was made by melting point determinations of the methyl esters of the porphyrins in the usual way. Most of the methyl esters were dried *in vacuo* over phosphorus pentoxide at room temperature, but a few were dried at 37.5°. As a check on the melting point, mixed melting points were determined in many instances—

TABLE II

Urinary Porphyrins

	Clinical diagnosis	Case No.	Urine collection		Porphyrin excretion			Porphyrin methyl ester	
			Duration	Amount	Total	Daily	Per liter	M.p.	Mixed m.p.
			days	*li-ters*	*mg.*	*mg.*	*mg.*	*°C.*	
Copropor-phyrin I	Catarrhal jaundice	1	18	20	10	0.56	0.5	245	+
	" "	2	15	22	12	0.8	0.55	252	+
	" "	3	10	10	5	0.5	0.5	247	+
	" "	4	11	13	3	0.27	0.23	246	+
	" "	5	15	10	3	0.2	0.3	243	+
	" "	6	12	10	4	0.33	0.4	230	+
	" "	7	15	20	7	0 4	0.35	249	+
	" "	8	13	15	4	0.3	0.27	248	+
	" "	9	8	10	2	0.2	0.2	242	
	Obstructive "	10	7	5	2	0.3	0.4	237	+
	" "	11		15	2			240	+
	Jaundice, passive congestion	12	28	37	4	0.1	0.1	240	+
	Atrophic cirrhosis of liver	13	34	20	6	0.18	0.3	243	+
	" "	14	22	15	5	0.2	0.3	248	+
	" "	15	16	22	5	0.3	0.2	246	+
	" "	16	7	10	5	0.7	0.5	247	+
	" "	17	6	6	2	0.3	0.3	238	
	" "	18		15	2			242	+
	Hemolytic jaundice	19	28	40	2	0.07	0.05	246	+
	" "	20-a	13	10	7	0.54	0.7	245	+
	" "	20-b	12	11	4	0.33	0.36	244	+
	" "	21	17	30	6	0.35	0.2	243	+
	" "	22	15	35	4	0.27	0.12	246	+
	" "	23						244	
	Hodgkin's disease	24	12	27	8	0.66	0.29	245	+
	" "	25	11	15	3	0.27	0.2	245	+
	" "	26	8	20	4	0.5	0.2	242	+
	" "	27		30	5			243	+
	" "	28	8	7	2	0.2	0.3	237	
	Lymphosarcoma of liver	29	40	35				241	
	Pulmonary abscess	30	10	15	2	0.2	0.1	248	+
	Lobar pneumonia	31	10	13	2	0.2	0.2	241	

TABLE II—*Concluded*

Clinical diagnosis		Case No.	Urine collection		Porphyrin excretion			Porphyrin methyl ester	
			Duration	Amount	Total	Daily	Per liter	M.p.	Mixed m.p.
			days	*liters*	*mg.*	*mg.*	*mg.*	*°C.*	
Uropor-phyrin I	Chronic porphyrin-uria	32		15				280	+
	" "	33	8	10	5	0.6	0.5	253	+
	Sulfonal poisoning	34		15	8			280	+
Copropor-phyrin III	Melanosarcoma of liver	35	30	40				146,168	
	Atrophic cirrhosis of liver	36	2	3	1	0.5	0.33	172	
	Pigment cirrhosis of liver	37	10	15	2	0.2	0.1	143,170	
Unknown porphyrin	" "	38-a	30	22	10	0.33	0.45	222	
	" "	38-b	20	8	4	0.2	0.5	222	

these are designated by the symbol + in the last column of Table II.

Results

The results are tabulated in Table II. The porphyrin excretion values were obtained for the most part by direct weighing of the methyl esters but a few were determined by a colorimetric procedure which will be described in a later publication. The data are not quantitative but serve as a qualitative measure only.

The melting points recorded are in all cases lower than those of the synthetic esters—coproporphyrin I methyl ester, 252°, uroporphyrin I methyl ester, 295°, coproporphyrin III methyl ester,[3] 142°, 172° (8). The observed differences are of the same order of magnitude as those obtained by other investigators working with natural porphyrins and are probably due to small amounts of

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[3] The methyl ester of coproporphyrin III has a double melting point. On determining its melting point it is found to melt first at the lower temperature, then after cooling, at the higher. The two melting points are represented as shown, *i. e.* 142°, 172°.

impurities, such as isomeric and other urinary porphyrins and traces of moisture.

In the instance of Case 20 the urinary porphyrin was characterized before (Case 20-a) and after (Case 20-b) splenectomy. Removal of the spleen had little effect on the porphyrin excretion.

With the uroporphyrin I isolated from the urine of the chronic porphyrinurias there was always present a small amount of ether-soluble porphyrin which was identical spectroscopically with coproporphyrin. Unfortunately the amount present was in all instances too small for melting point determination.

In Case 38 a new ether-soluble, chloroform-insoluble porphyrin was encountered. This porphyrin was isolated from the urine of a case of pigment cirrhosis of the liver. Its spectrum differed from those of the usual biological porphyrins—copro-, deutero-, uro-, and protoporphyrin—and the methyl ester gave depressions in mixed melting point determination with esters of coproporphyrin I and deuteroporphyrin IX. After five recrystallizations the melting point was raised from 218° to 222° and subsequent recrystallization did not further elevate it. 4 months after the first examination another specimen of urine (Case 38-b) was examined and found to contain the same porphyrin and a very small amount of a chloroform-soluble porphyrin.

DISCUSSION

As the results in Table II show, coproporphyrin is excreted in the majority of the pathological conditions examined. Fink (9) has recently characterized the normal urinary porphyrin as coproporphyrin I, which, according to Brugsch (10), is excreted at a daily level of 30 to 50 micrograms. The increased excretion of this normal constituent of the urine may denote either a condition of increased pigment metabolism, inability of the liver to excrete porphyrins, or a pathological change in the pigment picture. It is difficult to evaluate these factors since one or more may apply to a given condition.

The formation of porphyrins of type I must involve a synthesis differing fundamentally from the synthesis of blood pigment (hemoglobin and its oxidation product, bilirubin, are derivatives of etioporphyrin III) for it is extremely improbable that the tissues can convert a porphyrin of type III to one of type I. That the

formation of type I porphyrins has no direct connection with that of hemoglobin is shown by the study of yeasts. Normally yeast grown on hemoglobin-free substrates produces hematin IX—the hematin obtained by degrading hemoglobin—and a smaller amount of coproporphyrin I. Under pathological conditions, however, there is a marked increase in the production of coproporphyrin I (14). Apparently a parallel situation exists in the body tissues, pathological conditions leading to an increased production of coproporphyrin I.[4] To be sure, these pathological symptoms are frequently accompanied by liver insufficiency which in turn diminishes the power of the body to excrete porphyrins through the bile. Nevertheless, the large increase in urinary porphyrins appears to be due more to increased production than to faulty excretion.

The diseases in which excretion of coproporphyrin III occurs— pigment cirrhoses (hemosiderosis) and liver tumor—warrant especial attention. They may be characterized by an abnormal degradation of hemoglobin or the coproporphyrin III may originate from a part of the cellular respiratory system. If the former is the source, then the liver doubtless plays an important part in the formation of this type of porphyrin.

The two cases in which uroporphyrin I was isolated are duplications of cases which have been reported by other workers (1, 6, 11, 13).

SUMMARY

In the majority of diseases characterized by increased excretion of porphyrins in the urine the porphyrin excreted is coproporphyrin I. Since coproporphyrin I is present in small amounts in normal urine, the increased excretion may be viewed either as heightening of a normal process or a failure of the tissues to complete a normal synthesis. In contrast a few liver diseases—pigment cirrhosis (hemosiderosis) and liver tumor—lead to the excretion of coproporphyrin III.

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THE DETERMINATION OF SOME AMINO ACIDS IN CRYSTALLINE PEPSIN

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In 1929 Northrop (1) isolated crystalline pepsin from commercial pepsin preparations. This was the second crystalline enzyme to be reported and the first crystalline proteolytic enzyme. Previously crystalline urease (2) had been reported and since then trypsin (3), amylase (4), trypsinogen (5), chymotrypsin (6), chymotrypsinogen (6), and carboxypolypeptidase (7) have been reported in crystalline form. So far the interest has been primarily in a determination of the physical properties and the activities of these compounds. Very few chemical analyses other than ultimate analyses have been made. Levene and Helberger (8) determined the distribution of the basic amino nitrogen by the Van Slyke method, and further than that there have been no determinations of amino acids in crystalline pepsin. The purpose of this investigation was the quantitative determination of some of the amino acids for which accurate methods are available.

EXPERIMENTAL

The crystalline pepsin was prepared by the method of Northrop (1) from Parke, Davis and Company 1:20,000 U.S.P. pepsin and recrystallized four times. The crystals were dissolved in sodium acetate and dialyzed for 36 hours in a Kunitz dialyzer at 10° against 0.0005 M acetate buffer of pH 4.65. The solution was filtered and an aliquot used for total nitrogen determination. The non-protein nitrogen was equivalent to 4 per cent of the total nitrogen. It was determined by taking a 2 cc. aliquot of the original solution and adding 10 cc. of hot trichloroacetic acid and

determining the total nitrogen in the filtrate. The amino nitrogen was estimated by the Van Slyke method and was equivalent to 1.01 per cent of the total nitrogen. The specific rotation ($[\alpha]_D^{24}$, pH 4.65) was $-83°$.

A volume of the solution (415 cc.) containing exactly 50 gm. of protein was poured with rapid stirring into 1600 cc. of boiling dilute sulfuric acid (1580 cc. of water plus 20 cc. of N H_2SO_4). The beaker was placed immediately in an ice-salt bath of $-10°$ and within 10 minutes the temperature was 25°. The mixture before cooling was 80° and the pH was 4.0. The precipitate was filtered, washed with water and acetone, and dried. 40 gm. of dried material were obtained and 10 gm. failed to precipitate. This was rather surprising, since it has been previously shown that pepsin is quantitatively precipitated at pH 4. The precipitated material, as well as the uncoagulable residue, and some of the original uncoagulated dialyzed solution have been analyzed and the results are recorded in Table I. The various methods used are the same as those employed by Calvery and Freyberg (9) and references need not be repeated here.

DISCUSSION

From Table I it can be seen that there are several striking facts in the analyses. In the first place the low basic nitrogen values of Levene and Helberger (8) have been confirmed by actual isolation of the basic amino acids. Comparisons with other protein analyses reported in the literature show no other true proteins with such small quantities of the basic amino acids in them. Accompanying this low basic nitrogen figure one would expect a very high amino nitrogen value after acid hydrolysis when expressed as percentage of the total nitrogen, and that is what was found. The value of 91 per cent is higher than any value previously reported in the literature. The high amide nitrogen value corresponds to the large amount of dicarboxylic amino acids present. The very low humin nitrogen value is a point of considerable interest, in view of the fact that the tryptophane content is relatively high. If the presence of carbohydrate or aldehyde-forming substances is requisite for the formation of humin in the presence of tryptophane, it seems quite certain that such substances are not constituents of crystalline pepsin. The rela-

tively high tyrosine, aspartic acid, and glutamic acid values are undoubtedly the most important factors in influencing the isoelectric point of the crystalline pepsin, which is far on the acid side of that of most proteins.

The difference in composition of the two fractions obtained by heat coagulation can only mean, it seems to us, either that during the process the pepsin partially digested itself so that some non-coagulable material was removed or that the short period of heat-

TABLE I

Summary of Analyses of a Solution of Crystalline Pepsin and of Two Fractions Obtained from It by Heat Coagulation at pH 4

The nitrogen values are calculated as percentages of the total N. The other values are calculated as percentages of the ash- and moisture-free protein.

	Original pepsin	Coagulated precipitate	Filtrate from coagulated pepsin
Total N..............................	15.4	15.2	15.4
Amino "	1.0		
" " (after acid hydrolysis)........	91.2	87.0	92.2
Humin "	0.2	Trace	Trace
Amide "	8.8	8.2	9.3
Tyrosine................................	10.3	8.3	11.5
Tryptophane............................	2.2	2.1	2.2
Cystine.................................	1.4	1.2	2.2
Arginine N	2.7	2.4	3.5
Histidine "	0.05	0.1	0.1
Lysine N...............................	2.1	2.5	2.0
Aspartic acid...........................	6.8	6.4	6.7
Glutamic acid..........................	18.6	18.9	18.0

ing at pH 4 caused a slight hydrolysis. In view of the striking difference in the tyrosine and cystine percentages, it is obvious that some change has occurred, which leaves a coagulated material different in composition from the original starting material. This is a point which should be considered in many cases of analysis of the heat-coagulable material from a protein solution. The usual assumption is that the coagulum has the same composition as the original protein in the solution from which it was obtained; from Table I it is quite evident that this is not the case.

SUMMARY

1. Crystalline pepsin has been analyzed for total N, amino N (before and after acid hydrolysis), humin N, amide N, tyrosine, tryptophane, cystine, arginine, histidine, lysine, aspartic acid, and glutamic acid.

2. During heat coagulation at pH 4 there was partial destruction of the pepsin molecule and on analysis of the coagulated material and the non-coagulable material for the above substances differences were observed.

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CRYSTALLINE EGG ALBUMIN*

III. FRACTIONATION OF PEPTIC HYDROLYSIS PRODUCTS BY DIALYSIS

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In the first paper of this series (1) the hydrolysis of crystalline egg albumin by pepsin, papain-hydrocyanic acid, and pancreatic proteinase (trypsin) and the subsequent action of some other enzymes on the hydrolysis products produced by these enzymes were studied. The peptic hydrolysis products became of particular interest, since one-third of the peptide linkages in the egg albumin were hydrolyzed by this enzyme. This meant that one of two things had occurred, either tripeptides were the only products formed as a result of complete hydrolysis of albumin by pepsin, or longer chains and dipeptides and possibly free amino acids were formed. The second paper of the series (2) contains evidence which points quite conclusively to the second of the above two possibilities. By fractionation of the hydrolysis products into five fractions strong evidence was obtained of the presence of dipeptides and of free amino acids. The present investigation is a study of fractions of peptic hydrolysis products obtained by dialysis. A preliminary report of the results here presented has previously been made (3).

EXPERIMENTAL

100 gm. of crystalline egg albumin were suspended in 0.3 per cent hydrochloric acid, 20 gm. of pepsin (Difco, 1:20,000) were added, and the solution was made up to 2000 cc. with 0.3 per cent

hydrochloric acid. After 3 hours in a thermostat at 30° with occasional shaking practically all of the protein was in solution. After 21 days under these conditions, the increase in amino nitrogen was 24.5 per cent of the total nitrogen and 5 days later became constant at 26.1 per cent. The entire digestion mixture was heated on a water bath to 85° for 10 minutes, cooled, and filtered. The dried coagulated material weighed only 2.1 gm. and was discarded. It has been found by repeated experiments that heating the peptic digest at pH 2 to 3 for 10 minutes at 85° produces no

TABLE I

Composition of Original Solution and Dialysates of Peptic Hydrolysate of Crystalline Egg Albumin

The protein values (in gm.) are calculated from the total nitrogen (gm.) and the cystine, tyrosine, and tryptophane are calculated as percentages of the protein, while the other values are calculated as percentages of the total nitrogen.

	Original solution	Dialysate I	Dialysate II	Residue
Protein..........................	83.1	30.7	18.7	29.9
Total N........	12.8	4.7	2.9	4.6
Amino N........................	31.9	35.8	33.6	26.9
" " (after acid hydrolysis)...	79.4	80.7	82.3	82.7
Amide "........................	7.9	8 3	8.3	8.2
Humin "........................	0.8	0.6	0.9	0.6
Cystine..........................	1.3	1.4	1.1	1.6
Tyrosine........................	4.6	5.0	3.2	5.0
Tryptophane....................	1.6	1.6	1.1	1.6
Arginine N......................	10.4	10.9	10.1	9.4
Histidine "	2.4	2.0	1.4	2.9
Lysine N..............	5.5	5.9	5.5	4.1

alteration in the number of free amino and carboxyl groups as determined both by the Van Slyke method and by titration. A pepsin control containing 20 gm. of pepsin was run simultaneously with the digest, and all values reported and discussed in this investigation have been corrected for the pepsin in the digest.

A part of the above hydrolysate containing 12.8 gm. of nitrogen equivalent to 83.1 gm. of protein was placed in a large collodion bag and dialyzed against 2 liters of distilled water for 12 hours. This was called Dialysate I. The residue was dialyzed against 2

liters of fresh distilled water for a second 12 hour period.   This was called Dialysate II and that remaining in the bag was designated as the residue.   The above fractions and the original solution have been analyzed for a number of constituents by methods in general use in this laboratory (4) and the results are recorded in Table I.

Some experimental findings not easily included in Table I should also be presented.   Since we believe that complete peptic hydrolysis of protein leaves few if any high molecular weight compounds, it seemed advisable to determine the ratio of amino nitrogen to total nitrogen in the "proteose" fraction produced by saturation of a solution of peptic hydrolysis products with ammonium sulfate. The precipitate (proteoses) obtained by this procedure was dissolved in water and freed from ammonia.   The nitrogen was equivalent to 14.6 per cent of the total nitrogen of the original solution and 17.7 per cent of it was amino nitrogen.   The filtrate from the "proteoses" was also freed from ammonia and found to contain 41.1 per cent of its total nitrogen as amino nitrogen. Even the "proteose" fraction cannot contain many peptides having more than five or six amino acids and this fraction is only a small part of the original.

Cystine, tyrosine, and tryptophane were determined in the original peptic hydrolysate before acid hydrolysis and after acid hydrolysis.   The values before acid hydrolysis were cystine 1.6 per cent, tyrosine 2.3 per cent, and tryptophane 0.6 per cent, while after hydrolysis the following values were obtained, cystine 1.3 per cent, tyrosine 4.6 per cent, and tryptophane 1.58 per cent.

DISCUSSION

During this investigation many interesting observations have been made, some of which are confirmations of the findings of Miss Lila Miller (5) who is making an investigation of the action of various enzymes on lactalbumin, while some of our findings have been confirmed in her investigation.   It seems advisable to emphasize some of these points for the benefit of others who are interested in studying peptic hydrolysis products.   In the first place, contrary to the general impression, toluene and the acidity of a peptic hydrolysate provide complete protection against the growth of organisms, and at the same time the acid concentration

is not sufficient to produce detectable changes in the amino or carboxyl groups over long periods either in the ice box or in the thermostat at 30°. Miller (5) has found this same thing true in the case of lactalbumin. Again as already pointed out, heating at 85° for a few minutes to stop the action of the enzyme also fails to alter the amount of amino nitrogen or carboxyl groups as determined by the general methods in use for these determinations.

In the course of this investigation, it was necessary to allow the original digest, after being heated and filtered, to stand in the ice box for 2 months. The solution remained unchanged so far as could be detected and no sediment separated. After dialysis, the fractions obtained were again allowed to stand in the ice box for 2 months. From Dialysate I, 565 mg. of tyrosine were isolated, which confirms the finding of Northrop (6), who isolated tyrosine from a self-digestion of pepsin. Although it is conceivable that all of this tyrosine could come from the pepsin present, it does not seem probable, since it would represent more than 75 per cent of the total tyrosine in the pepsin, and furthermore only 150 mg. of tyrosine separated in the pepsin control. Therefore, *it seems to us quite certain that peptic hydrolysis liberates tyrosine from the egg albumin molecule, as well as from pepsin itself.* The only other reports of the liberation of free amino acids as a result of peptic hydrolysis, so far as we are able to find, are those of Felix (7), who reported the isolation of lysine from a peptic hydrolysate of histone, and Lieben and Lieber (8) who isolated free arginine from several proteins.

The results reported in Table I show that within 12 hours about one-third of the peptic hydrolysis products had passed through the membrane and within the next 12 hours almost half of the remaining products dialyzed. During this process 4.52 per cent of the total nitrogen and 4.53 per cent of the amino nitrogen were lost, probably due entirely to mechanical manipulations. Fortunately, the process did furnish some very definite and specific information, particularly the isolation of free tyrosine.

SUMMARY

1. Dialysis can be used for separation of peptic hydrolysis products into fractions of different composition.

2. Free tyrosine was isolated from a peptic hydrolysate of

crystalline egg albumin which, along with the isolation of tyrosine by Northrop from a peptic self-digestion and the isolation of lysine from a peptic hydrolysate of histone by Felix, leads to the conclusion, contrary to that generally accepted, that free amino acids are liberated when pepsin acts on protein.

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CRYSTALLINE EGG ALBUMIN

IV. THE RATE OF LIBERATION OF AMINO NITROGEN AND CYSTINE, TYROSINE, AND TRYPTOPHANE COLORIGENIC VALUES DURING PEPTIC, ACID, AND ALKALINE HYDROLYSIS OF EGG ALBUMIN

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In a recent investigation of the digestibility of proteins *in vitro*, Jones and Gersdorff (1) studied the rate of the liberation of cystine during peptic and acid digestion of casein. The Sullivan (2) method did not indicate any liberation of free cystine during peptic hydrolysis, while with acid hydrolysis it reached a maximum at 0.33 per cent at the end of 6 hours and remained constant during the extent of the experiment. Results by the Folin and Marenzi (3) method were quite different.

Jones and Gersdorff have further discussed at some length the liberation of amino acids during enzymic digestion of proteins, and they quote Abderhalden (4) as the only evidence that free amino acids are not liberated by the action of pepsin on protein. Since the work of Abderhalden several investigators have reported the isolation of free amino acids from peptic hydrolysates. Felix (5) has isolated free lysine from a peptic hydrolysate of histone, Northrop (6) isolated tyrosine from peptic self-digestion, Calvery and Schock (7) isolated tyrosine from a peptic hydrolysate of crystalline egg albumin, and Lieben and Lieber (8) have reported the presence of free arginine following peptic hydrolysis of several proteins. Although free amino acids may be liberated by peptic hydrolysis of protein, cystine does not seem to be one of them.

The present investigation is a study of crystalline egg albumin similar to that made by Jones and Gersdorff (1) of casein and includes the determination of tyrosine and tryptophane during

peptic and alkaline hydrolysis and the amino nitrogen during peptic hydrolysis.

The albumin was prepared according to the method of Sörensen and Höyrup (9) and recrystallized twice. A final volume of 2000 cc. of protein solution was obtained containing 18.9 mg. of albumin per cc.

Peptic Hydrolysis of Albumin—1000 cc. of the above solution (18.9 gm. of protein) were taken, enough hydrochloric acid was added to make a 0.3 per cent solution, and 1.89 gm. of pepsin (Difco, 1:20,000) were added. A control containing only pepsin was run at the same time. Since, as pointed out in an earlier investigation, digestion is so rapid at the beginning of the experiment and initial values are so difficult to obtain, the first values recorded in Table I are the values at the end of 15 minutes in all cases. The digestion was carried out at 30° in a thermostat and samples were removed at definite intervals. The amino nitrogen was determined by the Van Slyke method and the cystine, tyrosine, and tryptophane were determined by the methods of Folin and Marenzi (3, 10).

Acid Hydrolysis of Albumin—To 320 cc. of the above albumin solution were added 180 cc. of concentrated sulfuric acid and the volume made to 500 cc. This made a solution of approximately 65 per cent sulfuric acid. In a more dilute solution a precipitate remained and the early determinations of cystine could not be made. Since the concentration of acid was so high, it was only heated to 85–95°. Samples were removed at the same intervals as during the peptic hydrolysis and only cystine was determined after the acid concentration was carefully adjusted to that of the Folin and Marenzi (3) determination.

Alkaline Hydrolysis of Albumin—This hydrolysis was carried out in large individual Pyrex test-tubes. 15 cc. of the albumin solution and 10 cc. of 50 per cent NaOH were thoroughly mixed and placed in a boiling water bath for the definite periods of time exactly comparable to those of the acid and peptic hydrolyses. The procedure was then exactly the same as that in the Folin and Marenzi (10) micromethod for tyrosine and tryptophane.

It has been previously definitely established that the nitrogen

content of crystalline egg albumin when dried at 105–110° and corrected for ash is 15.4 per cent (11).   The total nitrogen was determined in the albumin solution used in this study and the albumin content calculated.   The amino nitrogen is expressed as percentage of the total nitrogen, while the chromogenic values are

TABLE I

Hydrolysis of Egg Albumin for Varying Lengths of Time

The amino nitrogen is expressed as percentage of the total nitrogen. All other values are expressed as percentages of ash- and moisture-free crystalline egg albumin.

Time	Amino N peptic hydrolysis	Chromogenic values calculated as					
		Cystine		Tyrosine		Tryptophane	
		Peptic hydrolysis	Acid hydrolysis	Peptic hydrolysis	Alkali hydrolysis	Peptic hydrolysis	Alkali hydrolysis
hrs.							
0.25	3.5	0.78	0.37	0.50	2.0	0.72	0.00
0.50	3.6	0.83	0.39	0.54	2.7	0.69	0.23
0.75	4.2	0.99	0.55	0.65	2.9	0.68	0.25
1.0	4.8	1.1	0.70	0.79	3.0	0.23	0.34
1.5	5.3	1.2	0.72	0.95	3.4	0.23	0.46
2.0	6.0	1.3	0.85	1.3	3.5	0.25	0.57
3.0	6.5	1.6	0.97	1.4	3.6	0.26	0.73
5.0	7.0	1.8	1.3	1.6	3.8	0.29	0.92
8.0	7.6	2.0	1.3	1.8	4.6	0.39	1.2
12.0	8.2	2.1	1.2	2.4	4.7	0.70	1.2
24.0	9.3	2.0	0.72	2.6	4.7	0.78	1.3
36.0	10	1.9	0.58	2.6	4.7	0.97	1.3
days							
4	13	1.8		2.7		1.0	
7	16	1.7		2.7		1.0	
14	20	1.7		2.7		1.1	
21	24	1.7		2.6		1.0	
36	25	1.6		2.6		1.0	

expressed as percentages of tyrosine, tryptophane, and cystine in the albumin present.   A summary of the results is incorporated in Table I.

DISCUSSION

The results in Table I show that the Folin and Marenzi values for cystine in egg albumin as determined in this investigation differ

quite markedly from those reported by Jones and Gersdorff (1) for casein.   In the acid hydrolysate the values for cystine gradually rose to a maximum of 1.3 per cent, following which there was gradual destruction of the cystine by the strong acid used.   This value of 1.3 per cent is the same as that previously·reported by one of us and by other investigators.   In the case of the peptic digest there was again no rapid rise at the beginning to a sharp peak but a slow rise to a value far above that found by acid hydrolysis. This has been previously found in other peptic digests (unpublished data).   Also the final value after 36 days is greater than that found by acid hydrolysis.

In confirmation of the findings of Jones and Gersdorff that 0.1 ɴ hydrochloric acid did not hydrolyze casein, it has been repeatedly demonstrated in this laboratory that 0.3 to 0.5 per cent hydrochloric acid has *very little* hydrolytic action on the native protein and *none* on the partial or final peptic hydrolysis products in the thermostat at 30° for long periods of time or when heated for 5 minutes at 85° (unpublished experiments of Miss Lila Miller and of ours).

In a very interesting paper published recently by Cohn and White (12) in an attempt to explain the early findings of Mendel and Lewis (13) it was found that cooked egg white was more readily digested than raw egg white.   As shown by this present experiment, in which a pepsin to albumin ratio of 1:10 was used, and as pointed out by Calvery (14) in an earlier investigation, pepsin readily attacks *uncoagulated crystalline egg albumin* and the digestion is so rapid during the first few minutes that duplicate Van Slyke determinations for amino nitrogen cannot be made to check each other.   It has further been often demonstrated in this laboratory that raw egg white as well as crystalline albumin can be readily digested by pepsin (unpublished data).   This does not necessarily contradict the findings of Cohn and White (12), since the conditions of experimentation are somewhat different.

SUMMARY

Uncoagulated crystalline egg albumin is readily hydrolyzed when the pepsin (Difco, 1:20,000) to protein ratio is 1:10 and the acid concentration is approximately 0.3 per cent.   During the digestion the amino nitrogen value gradually rises to a maximum

of 25.1 per cent in 36 days, while the chromogenic value obtained by the Folin and Marenzi method for cystine rises gradually to a peak of 2.1 per cent within 12 hours and then falls to a constant value between 1.6 and 1.7 per cent during the next 35 days. This is much higher than the highest value of 1.3 per cent obtained by acid hydrolysis in this experiment and the same value obtained previously by other investigators. The acid peak was reached after a gradual rise and did not follow the same curve at all as that obtained by Jones and Gersdorff (1) for the cystine content of casein.

The tyrosine and tryptophane colorigenic values obtained by the Folin and Marenzi micromethod during both peptic and alkaline hydrolysis rose gradually until they reached maximum values. The values were not the same during peptic hydrolysis as those obtained during alkaline hydrolysis. A discussion of the values obtained and their significance is included.

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STUDIES IN HISTOCHEMISTRY*

V. THE VITAMIN C CONCENTRATION OF THE CORPUS LUTEUM WITH REFERENCE TO THE STAGE OF THE ESTROUS CYCLE AND PREGNANCY†

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The singularly high concentration of vitamin C in certain physiologically related endocrine organs is striking. Of all animal tissues studied, the adrenal cortex, corpus luteum, and anterior lobe and pars intermedia of the hypophysis are the richest sources; and of these, the pars intermedia contains vitamin C in the greatest concentration (4).

The present paper continues our earlier histochemical studies of vitamin C distribution in mammalian endocrine organs (2, 4). The correlation between the vitamin C concentrations of functionally related portions of the adrenal and hypophysis (4) may be extended to the corpus luteum, which is physiologically related to the anterior lobe of the hypophysis and contains vitamin C in approximately the same concentration.

That this parallelism between vitamin C concentration and function is not merely a coincidence seems to be borne out by the work of Kramer, Harmon, and Brill (5) who have reported degeneration of both follicles and corpora lutea in scorbutic guinea pigs, as well as failure to become pregnant or to deliver normal young. Ingier had shown that on scorbutic diets in early pregnancy the animals were born dead or prematurely (6). In this connection there should be mentioned a recent note by Bourne (7) who reported

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* For the previous papers of this series see (1–4) respectively.

† Included in a paper presented before the vitamin symposium at the meeting of the American Chemical Society at San Francisco, September, 1935.

that pregnant guinea pigs remain normal on a scorbutic diet. As will be pointed out later, there may be a connection between the presence of vitamin C and the corpus luteum hormone, progesterone.[1]

Vitamin C in the corpus luteum has been reported by Bessey and King (9) who found 1.39 mg. per gm. in this body in the sow, and Huszák (10) who reported that an early corpus luteum in the pig had a concentration of 0.74 mg. per gm., while an involuted body had only 0.19 mg. per gm.; Huszák also reported other corpora lutea of pigs varying from 1.02 to 1.50 mg. per gm., and one from a cow with 1.19 mg. per gm. Giroud, Leblond, and Giroux (11), using the silver nitrate staining method for the identification of vitamin C, found a strongly positive reaction for corpora lutea of estrus and pregnancy. However, as previously mentioned, the unreliability of the staining reaction is noteworthy (1, 2).

Except for the two pig corpora lutea of Huszák referred to above, no attempt was made in any of this previous work to correlate the vitamin concentration with the exact stage of the cycle of the corpus luteum.

In our earlier work on the histochemical distribution of vitamin C in endocrine organs (2, 4) we determined the concentration of the vitamin, as well as the numbers of each type of cell in the various portions of the organ in question, so that the vitamin content per cell might be found. In the present case, however, this was not done. Since the corpus luteum is not a layered body but contains a homogeneous mixture of several types of cells, no cell counts were made, since counts of several types of cells could contribute nothing to the estimation of the vitamin concentration in any one type of cell. Hence only the concentration of vitamin C in corpora lutea as a whole at various stages of estrus and pregnancy was determined.

EXPERIMENTAL

Because of their convenient size, availability of the normal tissue in the fresh state, and for consistent comparison with the other bovine endocrine glands studied, corpora lutea of the cow

[1] The recently approved term "progesterone" will be used throughout this paper (8).

were employed. Another advantage derived from using the bovine corpus luteum is that it resembles the human more than does that of the pig, sheep, and other animals, since only one corpus luteum is formed during each estrous cycle. The ovaries were removed from the freshly killed animals, stored at −5° as previously described (2), and used for vitamin C titration within 1 to 2 days.

Though macroprocedure could have been employed for the vitamin C estimation in the corpora lutea, the micromethod previously employed (1) was used instead, since it offered the advantage that many analyses could be performed on each corpus luteum and enough tissue for histological study still be left. A further benefit was conservation of reagents and time. As many as 50
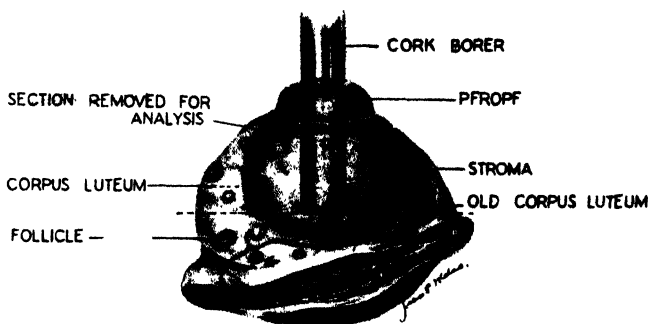
Fig. 1. Method of sampling corpus luteum in bovine ovary (approximately actual size).

separate titrations could be performed in the course of 2 to 3 hours by this method. Each c.mm. of the standard 2,6-dichlorophenol indophenol solution employed for the titration was equivalent to 0.130 microgram of vitamin C.

The sampling and sectioning of the tissue were conducted in a manner similar to that previously employed (2, 4). Fig. 1 demonstrates the manner in which the samples were removed. The stiffly frozen ovary was first cut in half along the horizontal middle dotted line, then the cork borer, which had an internal diameter of 4.2 mm., was pushed through the corpus luteum in the manner indicated. The cylinder of tissue thus removed was placed at once on a freezing rotary microtome, a drop of normal

saline, rather than water (2), being used to make firm contact between the tissue and the freezing block. In order to obtain representative sections (30 μ thick) of tissue for vitamin C extraction and titration, two adjacent sections were removed for two separate analyses, after which 50 sections were discarded, and then two more taken for vitamin estimation, 50 again discarded, and so on through the entire cylinder of tissue. The homogeneity of the corpus luteum is emphasized by the consistent titration results obtained. Usually ten titrations were performed on each sample of tissue; *i.e.*, duplicate determinations in five regions 1500 μ apart. The block of tissue surrounding the hole left by the cork borer was fixed in Bouin's fluid, mounted in paraffin, sectioned, and stained with iron hematoxylin, acid fuchsin, and light green.

The approximate age of the corpus luteum could be estimated from the gross and histological appearance. In the case of corpora lutea of pregnancy, the size of the fetus was used as the chief criterion of the age of the corpus luteum and the duration of pregnancy. The relation of the age of a fetus to its size was obtained according to Hammond (12).

Changes Occurring in Corpus Luteum Since it was impossible for us to have cows killed at known stages of their estrous cycles, it became necessary to determine the stage by macroscopic and microscopic examination of the organs. The following is a résumé of the changes that occur.

The macroscopic and microscopic changes have been described by Hammond (12). When the Graafian follicle bursts and discharges its ovum, the granulosa cells of the lining of the collapsed follicle rapidly become transformed into large pigmented luteal cells which resemble the cells of the adrenal cortex. The luteal cells enlarge, and the corpus luteum as a whole increases in size until it reaches a maximum diameter of about 2.0 cm. (in pregnancy the size is a little larger). This maximum is reached approximately 6 to 8 days after ovulation. In the normal estrous cycle of 21 days, involution sets in after the 15th to 17th day and the corpus regresses and steadily diminishes in size until it finally leaves a pigmented scar in the ovary. At the time of the next ovulation the size of the corpus has decreased to about half the maximum size. In pregnancy the maximum size and full function are maintained up to parturition. Atrophy is delayed until about 30 days after parturition.

TABLE I

Vitamin C Content of Bovine Corpus Luteum at Various Stages of Estrous Cycle

Corpus luteum No.	24	26	31	10	3	2	14	32	13	33	9	8
Size, cm.	0	2.0	2.0	2.0	1.9	2.0	1.6	1.7	1.0	0.6	0.5	0.9
No. of sections analyzed	10	10	10	9	11	10	11	8	18	10	3	5
Average vitamin C per section, *microgram*	0.58	0.58	0.54	0.50	0.48	0.44	0.41	0.23	0.16	0.17	0.14	0.13
Maximum deviation from mean, vitamin C per section, *microgram*	+0.06 / −0.07	+0.06 / −0.08	+0.09 / −0.08	+0.03 / −0.02	+0.11 / −0.06	+0.07 / −0.08	+0.09 / −0.05	+0.08 / −0.08	+0.02 / −0.01	+0.02 / −0.02	+0.02 / −0.01	+0.01 / −0.01
Average concentration, *mg. per gm.*	1.4	1.4	1.3	1.2	1.2	1.1	1.0	0.56	0.39	0.41	0.34	0.31
Age groups	B	B	B	B	B	C	C	C	D	D	D	D

TABLE II

Vitamin C Content of Bovine Corpus Luteum in Various Stages of Gestation

Corpus luteum No.	22	23	21	19	25	20	18	29	17	28	27	30	16	11
Size, cm.	2.0	2.0	2.2	2.0	1.8	2.0	2.1	2.0	2.2	2.3	1.7	1.8	2.1	2.4
No. of sections analyzed	13	10	10	10	7	11	9	10	10	10	7	8	12	13
Average vitamin C per section, *microgram*	0.68	0.74	0.90	0.91	0.90	0.68	0.67	0.71	0.62	0.75	0.79	0.83	0.73	0.46
Maximum deviation from mean, vitamin C per section, *microgram*	+0.11 / −0.06	+0.06 / −0.10	+0.01 / −0.04	+0.06 / −0.07	+0.09 / −0.07	+0.03 / −0.03	+0.03 / −0.03	+0.03 / −0.03	+0.05 / −0.06	+0.02 / −0.03	+0.02 / −0.03	+0.07 / −0.09	+0.04 / −0.04	+0.03 / −0.04
Average concentration, *mg. per gm.*	1.6	1.8	2.2	2.2	2.2	1.6	1.6	1.7	1.5	1.8	1.9	2.0	1.8	1.1
Approximate age, *mos.*	1.6	2	2	2.2	2.2	2.6	2.8	3.8	4.4	4.4	6	6.6	7.2	8
Size of fetus, *cm.*	4	5	5	6	6	10	12	22	30	30	50	60	70	80

During regression the luteal cells degenerate, the blood sinusoids increase in size and number, the walls of the blood vessels thicken, and filaments of fibrous tissue increase to thick bands.

Results

The corpora lutea of estrus were divided into four age groups. Group A was composed of organs just forming (up to 5 or 6 days old), Group B at the maximum development (from 5 or 6 to 16 or 17 days), Group C at the beginning of involution (from 16 or 17 to 23 or 24 days), and Group D with further involution and atrophy (all corpora older than 24 days). No corpora lutea could be obtained from non-pregnant cows that we could be sure fitted into Group A.

The ovarian tissue surrounding a corpus luteum (No. 1, Table I) was analyzed for vitamin C and found to contain 0.32 mg. per gm.

DISCUSSION

It may be seen from Table I that the vitamin C content of the corpus luteum is at a maximum when the organ itself is fully mature, and decreases as the corpus involutes and atrophies. Since this body is in full function throughout pregnancy, it is not surprising that the vitamin concentration is at a rather constantly high level during the course of gestation (Table II). It is interesting to note that the corpus luteum of pregnancy has a concentration of vitamin C practically 50 per cent higher than the mature corpus in the non-pregnant animal.

A comparison of old atrophic corpora lutea with normal ovarian tissue shows that the two have practically the same vitamin C concentration, the former having 0.31 to 0.39 mg. per gm., while the latter contains 0.32 mg. per gm.

Vitamin C appears to be unrelated to the female sex hormone, estrin, since follicular fluid is very poor in the vitamin (9, 10) and the estrin content of corpora lutea of gestation falls off almost to zero towards the end of the pregnancy ((13) p. 417), though it has been shown in the present paper that the vitamin C content remains at a high level. On the other hand, vitamin C may be related to progesterone. The variations in the vitamin content of the corpus luteum that we have found seem to parallel the

progesterone content (14). The necessity for the presence of a corpus luteum for proper embryo implantation and continuance of gestation depends upon the progesterone it produces (13, 15). When it is recalled that vitamin C deficiency produces degeneration of corpora lutea and failure of normal gestation in guinea pigs (5), it would appear at least reasonable to suggest that the vitamin is necessary for the normal production of the hormone, either by maintaining the integrity of the structures responsible for its formation or more directly by influencing the chemical reactions involved in the progesterone synthesis. Bourne (16) has suggested that vitamin C is associated non-specifically with the production of the corpus luteum hormone.

SUMMARY

The vitamin C content of corpora lutea of cows was determined in various stages of the estrous cycle and gestation. The concentration of vitamin varied with the degree of development of the corpus luteum of estrus, being at a maximum of 1.4 mg. per gm. of tissue when the organ was most fully developed and falling off to 0.3 mg. per gm. with regression.

The vitamin level in gestation remained at from 1.5 to 2.2 mg. per gm. for the first 7 months, decreasing to 1.1 mg. per gm. in the 8th month.

The relation of vitamin C to progesterone was indicated.

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NITROGEN AND SULFUR METABOLISM IN SUPRARENALECTOMIZED RATS

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Loeb (1) and Harrop (2) and their coworkers have presented evidence that the suprarenal cortical hormone exerts a regulatory influence upon the kidney. Their work was done on dogs which invariably died after removal of the suprarenal glands if no substitutional therapy was instituted. For extended metabolic studies rats are more suitable, since they usually possess sufficient microscopic accessory suprarenal cortical tissue to allow for long survival periods after suprarenalectomy, if sufficient attention is given to operative technique, after-care, and diet. As has been emphasized in a recent review by Perla and Marmorston (3), the high mortality rate frequently encountered in the literature (4–6) seems to be due mostly to non-observance of these factors. All our rats survived without treatment for the duration of the experiment (55 days after operation), when they were killed. No macroscopic suprarenal tissue was found at autopsy.

That this high survival rate is not due to a colony difference (7) is shown by the fact that several groups of suprarenalectomized rats died when kept on a diet adequate for normal rats, though somewhat lacking in vitamin content, while on an improved diet all of them survived. This agrees with the findings of Estrada (8), who reported that suprarenalectomized rats require more vitamin B than normal animals. In order to be able to differentiate clearly between the effects of diminished food intake and of removal of the suprarenal glands, paired feeding was resorted to. These controls showed none of the changes found in the nitrogen and sulfur metabolism of suprarenalectomized animals.

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Methods

Thirty-two male and female albino rats were used in the experiment. At the time of suprarenalectomy they were about 3 months of age. The rats were kept in groups of four in metabolism cages, as described in a previous publication (9). The diet consisted of our standard diet to which were added 10 per cent of whole milk powder, 5 per cent of dry yeast specially rich in vitamin B, and 2 drops per cage of concentrated fish oil[1] containing 60,000 units of vitamin A and 8500 units of vitamin D per gm.

Urine and feces were collected twice a week. The urine was analyzed for total nitrogen (Kjeldahl), urea (10), ammonia (Folin), uric acid (11), creatine and creatinine (12), total sulfur (13), total and inorganic sulfates (Folin), and copper (14). The feces, as well as the food, were analyzed for total nitrogen (Kjeldahl), total sulfur (15), copper (14), and iron (16). During the first and second period after suprarenalectomy (of 9 and 18 days respectively) urine and feces were analyzed in 3 or 4 day periods. Maximum, minimum, and average figures only are reported for these periods.

The rats were studied for a control period of 35 days before and 55 days after suprarenalectomy. During the first 9 days after operation there is a loss in weight, the food intake is diminished, and pronounced changes occur in nitrogen and sulfur metabolism. Then follows a period of 18 days when the food intake improves somewhat, but while the animals still lose weight, the changes in N and S metabolism become less pronounced. In the last period of 28 days the food intake is higher than during the control period and there is a gain in weight, but in spite of this the changes in N and S metabolism become again quite as pronounced as they were immediately after suprarenalectomy. Since no change occurred in the copper and iron metabolism, protocols are presented only for nitrogen and sulfur metabolism.

DISCUSSION

Silvette and Britton (17) state that suprarenalectomized rats excrete less urine than normal animals and that their ability to excrete injected fluids is markedly reduced. Under the conditions of our experiment we have observed no such impairment of

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[1] We are indebted to Mead Johnson and Company for supplying the oil.

the ability of the kidney to excrete water, but rather a diuresis, which is in accord with the work of Loeb and associates (1), who found an increase in urine volume in dogs after suprarenalectomy.

TABLE I

Nitrogen Metabolism (Daily Average per Rat)

Date	Urine	Feces	Total N Urine	Per cent of intake	Feces	Total excretion	Intake	Retention	Per cent of intake	Urea N	NH₃-N	Uric acid N	Preformed creatinine	Creatine	pH
1935	*cc.*	*gm.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Mar. 26–Apr. 1...	2.5	1.1	106		82	188	371	183		79	7.9	0.5	5	1	6.4
Apr. 2– 8.......	1.6	1.3	110		85	195	433	238		77	8.7	0.5	5	1	6.4
" 9–15.......	1.9	1.4	103		83	186	411	225		75	6.4	0.5	4	1	6.4
" 16–22.......	2.3	1.2	103		79	182	393	211		76	9.8	0.4	5	1	6.4
" 23–29.......	2.8	1.4	118		88	206	405	199		79	10.3	0.4	5	1	6.4
Mar. 26–Apr. 29..	2.2	1.3	108	27	83	191	403	212	52	77	8.6	0.5	5	1	6.4
May 1–9*															
Maximum......	10.5	1.3	189		81	270	377	132		136	11.7	0.6	6	10	7.4
Minimum......	3.0	0.9	127		55	182	314	107		96	9.0	0.4	5	5	7.4
Average........	7.4	1.1	162	47	71	233	349	116	33	121	10.3	0.5	6	8	7.4
May 10–27															
Maximum......	7.6	1.7	155		120	275	389	172		136	10.7	0.6	6	6	7.4
Minimum......	4.8	1.1	132		70	202	374	114		116	7.5	0.3	5	5	7.4
Average........	5.8	1.2	146	38	89	235	383	148	39	124	8.9	0.5	6	5	7.4
May 28–June 3...	10.3	1.4	182		90	272	386	114		154	10.5	0.5	6	6	6.8
June 4–10.......	11.6	1.6	234		96	330	462	132		198	10.4	0.4	6	7	6.8
" 11–17.......	9.0	1.6	194		91	285	421	136		170	9.0	0.5	6	6	6.6
" 18–24.......	10.7	1.7	230		99	329	462	133		189	9.4	0.5	6	6	6.6
May 28–June 24..	10.4	1.6	210	49	94	304	433	129	30	178	9.8	0.5	6	6	6.7

* Suprarenalectomized on April 30.

That this is not due to a species difference seems to be supported by the findings of Harrop and coworkers (2), who found no appreciable changes in the water content of liver and muscle tissues of either suprarenalectomized dogs or rats, while Silvette and Britton

(17) report that the liver and body tissues of their suprarenalectomized rats show a higher water content than those of normal animals.

We find that suprarenalectomized animals show a diminution of the ability to concentrate nitrogen (Table I). Nitrogen retention decreased from 52 per cent of the intake during the control period to 33 per cent and 39 per cent respectively during the following two periods. The food intake for these periods was lowered but not sufficiently to account for the drop in retention. And even during the last period when the food intake was increased, retention did not improve, but remained at the low level of 30 per cent of the intake. Putschkow and Krasnow (18), in their studies on suprarenalectomized cats and dogs, find a decrease in urea nitrogen in the urine, indicating impairment of liver function. In rats we find that contrary to the findings in cats and dogs the urea-forming ability of the liver is fully preserved. The urea content of the urine even rises from 71 per cent of the total urinary nitrogen during the control period to 85 per cent after suprarenalectomy. The increase in nitrogen excretion seems to be due to impairment of the concentrating power of the kidney, since the nitrogen concentration per 100 cc. of urine falls from 4.9 gm. of nitrogen during the control period to 2 gm. of nitrogen after suprarenalectomy. The diuresis found in suprarenalectomized animals is much more pronounced than that in normal animals when the kidney is called upon to excrete an unusually heavy load of nitrogen. There diuresis tends to regulate the volume of urine in such a way as to keep the nitrogen concentration constant. Jackson and Riggs (19) in their studies on the influence of high protein diets on the kidneys of rats have shown that the resultant diuresis was of such an order as to take care of the increased amount of nitrogen to be excreted, while keeping the nitrogen concentration at 4.5 gm. of nitrogen per 100 cc. of urine, exactly as it had been on their standard diet.

One of the most striking changes in nitrogen metabolism is the pronounced creatinuria encountered in suprarenalectomized rats. The muscular weakness occurring after suprarenalectomy has been shown to be due to a disturbance in phosphagen metabolism by Ochoa and his coworkers (20). They suggest that the decreased capacity for the performance of work is due to a diminished

capacity of the muscle to resynthesize phosphagen. But only the muscles of animals that present definite clinical symptoms of

TABLE II

Sulfur Metabolism (Daily Average per Rat)

Date	Total S								Neutral S	Per cent of urinary S excretion	Inorganic sulfates	Per cent of urinary S excretion	Ethereal sulfates
	Urine	Per cent of intake	Feces	Per cent of intake	Total excretion	Intake	Retention	Per cent of intake					
1935	*mg.*		*mg.*		*mg.*	*mg.*	*mg.*		*mg.*		*mg.*		*mg.*
Mar. 26–													
Apr. 1.....	7.3		8.0		15.3	35.4	20.1		0.5		6.2		0.6
Apr. 2–8	7.4		9.2		16.6	41.4	24.8		0.2		6.6		0.5
" 9–15....	7.3		10.8		18.1	39.3	21.2		0.7		6.1		0.5
" 16–22....	7.8		9.3		17.1	37.5	20.4		0.7		6.6		0.5
" 23–29....	9.3		10.6		19.9	38.7	18.8		1.2		7.6		0.6
Mar. 26–													
Apr. 29...	7.84	20	9.57	25	17.41	38.5	21.1	55	0.67	8.5	6.64	85	0.54
May 1–9*													
Maximum...	14.99		8.79		23.78	36.0	14.4		3.71		10.98		0.52
Minimum...	9.28		6.31		15.59	30.0	12.2		1.80		6.96		0.20
Average.....	12.55	38	7.90	24	20.45	33.3	12.8	38	2.73	22	9.48	76	0.34
May 10–27													
Maximum...	10.74		12.16		22.90	37.2	19.2		1.48		8.48		1.19
Minimum...	9.52		7.01		16.53	35.7	14.3		0.74		7.53		0.40
Average.....	10.08	28	9.09	25	19.17	36.6	17.4	48	1.13	11	8.19	81	0.76
May 28–													
June 3....	14.2		9.5		23.7	36.9	13.2		2.6		11.3		0.3
June 4–10....	17.0		11.0		28.0	44.1	16.1		2.5		13.7		0.9
" 11–17....	14.4		11.7		26.1	40.2	14.1		2.6		11.2		0.6
" 18–24....	15.5		11.6		27.1	44.1	17.0		2.5.		12.7		0.3
May 28–													
June 24...	15.28	37	10.93	26	26.21	41.3	15.1	37	2.54	17	12.23	80	0.51

* Suprarenalectomized on April 30.

suprarenal insufficiency show this phenomenon, while the breakdown of phosphagen in the muscles of animals that show no defi-

nite symptoms of insufficiency is negligible (21). The common factor in all conditions where creatinuria occurs is a disturbance in carbohydrate metabolism. It is well known that in suprarenalectomized animals the ability of the liver to store glycogen is impaired, though the longer an animal survives, the more glycogen is left in the liver (22). Presumably this lack of liver glycogen is a determining factor in the disturbance of the creatine metabolism of suprarenalectomized rats.

It has been suggested that the suprarenal glands are concerned in the regulation of sulfur metabolism. Loeper (23) found an increase in sulfur in the blood of patients suffering from Addison's disease. The proportion of oxidized sulfur was reduced, while that of neutral sulfur was increased. The suprarenal glands retain part of the blood sulfur, especially neutral sulfur. Loeper and coworkers also showed (24) that the neutral sulfur in the suprarenal glands of guinea pigs can be increased by injections of sulfur or of substances from which sulfur can be split off. Swingle and Wenner (25) also found a rise in inorganic blood sulfate when serious symptoms of suprarenal insufficiency appeared in cats and dogs.

As shown in Table II, the urinary sulfur excretion increases from 20 per cent to 38 per cent of the intake after suprarenalectomy. This increase takes place in the period in which the sulfur intake is decreased, as well as in the last period when the intake increases. While the percentage of sulfate excretion drops slightly, the neutral sulfur is increased from 8.5 per cent to 22 per cent of the total urinary sulfur excretion, indicating a disturbance in endogenous sulfur metabolism, which is in accord with the observations on the relation of the suprarenal glands to sulfur metabolism recorded in the literature.

SUMMARY

The nitrogen, sulfur, copper, and iron metabolism of suprarenalectomized rats has been studied. Fecal nitrogen excretion remains unchanged. Urinary nitrogen excretion increases, but the ability of the kidney to concentrate nitrogen is impaired. Urea excretion increases, with little change in ammonia excretion. Uric acid remains unchanged. There is a pronounced creatinuria. Fecal sulfur excretion remains unchanged. Excretion of total

urinary sulfur increases, as well as that of neutral sulfur. The percentage of sulfate excretion drops slightly. There is no change in copper and iron metabolism.

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CONDENSATION PRODUCTS OF ACETOACETIC ESTER

IV. TWO HIGHLY REACTIVE COMPOUNDS OF GLUCOSE AND ACETOACETIC ESTER

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The senior author has previously described a number of condensation products of acetoacetic ester and acid which are more easily oxidized *in vitro* than acetoacetic ester or acid (1). Some of the derivatives of glucose were found to be especially unstable and easily oxidized, and the point was made that analogous compounds may be involved in the mechanism of antiketogenesis, according to the theory of Shaffer (2). As a result of further studies two isomeric and more reactive compounds of the series have been prepared. When glucose cycloacetoacetic ester (3) is oxidized with mercuric sulfate at room temperature, a non-crystalline, glassy, hygroscopic substance is obtained which is exceedingly unstable in alkaline solution and is a powerful reducing agent. It is characterized by instantly turning a red-brown color upon treatment with alkalies. It reduces Fehling's solution quickly in the cold, and rapidly reacts with phenylhydrazine to give an oily liquid which quickly decomposes to a tar. It readily reacts with hydroxylamine to form an unstable product. The compound yields iodoform instantly when treated with alkaline hypoiodite. It resinifies and decomposes when kept in a desiccator over sulfuric acid. The compound has the elementary composition $C_{12}H_{18}O_8$. There are three hydroxyl groups in the molecule and one CH_3CO group. Benzoylation yielded a dibenzoyl derivative containing one hydroxyl group, as shown by the pyridine-acetic anhydride titration procedure. Apparently one hydroxyl group is less active than the remaining two.

A compound, isomeric with the above substance, was obtained by oxidizing glucose cycloacetoacetic ester with perbenzoic acid in the presence of $BaCO_3$. The product resembles that from the $HgSO_4$ oxidation in physical appearance and solubility, but differs from it in optical rotation and reacts more slowly with alkaline hypoiodite. Also it contains only two hydroxyl groups reactive toward acetic anhydride in the presence of pyridine in the cold, whereas the $HgSO_4$ oxidation product contains three such groups. The isomers behave similarly toward the action of alkali and Fehling's solution.

Work relating to the structures and physiological actions of these and similar compounds is in progress.

<div align="center">EXPERIMENTAL</div>

Oxidation of Glucose Cycloacetoacetic Ester with Mercuric Sulfate—20 gm. of glucose cycloacetoacetic ester (3), 60 gm. of mercuric sulfate, and 100 cc. of water were vigorously stirred at room temperature for 1 hour. 200 cc. of water were added and the precipitate was filtered off. The filtrate was shaken with 65 gm. of $BaCO_3$ until evolution of CO_2 ceased. The precipitate was filtered off, washed with 25 cc. of water, the combined washing and filtrate cooled to 0–5°, and 30 gm. of Zn dust added to remove mercury. After filtration the solution was treated with $BaCO_3$ (70 gm.) until no more CO_2 escaped, the precipitate was filtered off, washed with water, and the solution treated with 15 gm. of oxalic acid and cooled in an ice bath for ½ hour. The zinc oxalate was filtered off and washed with water. The solution was shaken with $BaCO_3$ until the liquid did not redden blue litmus. The precipitate was filtered off and washed with water. The filtrate (about 400 cc.) was concentrated as much as possible at the water pump at 50°. The heavy syrup was taken up in dry acetone, leaving an insoluble yellow precipitate. Acetone was added until no further precipitate formed. After cooling in an ice bath the precipitate was filtered off and the acetone removed from the solution as thoroughly as possible by a current of dry air in a bell jar. The last traces of acetone were removed at the oil pump, the heavy syrup becoming a voluminous yellow friable mass which was powdered. Yield 8 to 10 gm. The material contained considerable ash. It was dissolved in 50 cc. of

absolute alcohol and precipitated by pouring, with stirring, into 200 cc. of dry ether. After three such treatments and removal of the solvent the ash content was reduced to about 0.40 per cent. The product is a pale yellow, exceedingly hygroscopic glass which can be handled only after it has been converted into a friable mass by evaporating a solvent from it. The compound should be kept in a desiccator over $CaCl_2$. It quickly resinifies over H_2SO_4. The glass melted over the range 65–72°. It is soluble in water, alcohol, and acetone in practically all proportions, only slightly soluble in chloroform and ether, and insoluble in petroleum ether.

$C_{12}H_{18}O_8$ (290)

Calculated. C 49.65, H 6.25, 3OH 17.57, OC_2H_5 15.52, CH_3CO 14.83
Found.   " 49.71, " 6.58, " 18.31,   " 15.10,   " 14.14

Hydroxyl was determined by the method of Peterson and West (4) with acetylation at $-2°$ for 72 hours. CH_3CO was determined as follows: 50 cc. of 0.2 N I_2 were added to the compound dissolved in 100 cc. of water. 25 cc. of 10 per cent NaOH were quickly added and mixed, followed by 25 cc. of 1:1 HCl after 1.5 minutes (temperature of solution 33°). The liberated iodine was titrated with 0.1 N thiosulfate and the value subtracted from a blank similarly treated. 0.1394 gm. of compound reacted with 27.43 cc. of 0.1 N I_2. Calculated 28.77.

$$[\alpha]_D^{25} = -70.9° \ (3.65 \text{ per cent in water}). \text{ No mutarotation}$$

Benzoylation of Oxidation Product—10 gm. of the oxidation product, 50 cc. of chloroform, and 50 cc. of dry pyridine were mixed and cooled in an ice bath. 13.5 cc. of benzoyl chloride were added drop by drop for 2 hours with vigorous stirring. After standing overnight at room temperature 4 cc. of water were added. After ½ hour the mixture was diluted with ice water and extracted five times with 50 cc. portions of chloroform. The extract was washed five times with 50 cc. of cold dilute H_2SO_4 followed by three washings with 50 cc. of cold water. Most of the chloroform was removed at the pump. The heavy syrup was dissolved in alcohol and poured into several volumes of water with stirring. After the precipitated syrup had coalesced, the water was decanted and fresh water added with stirring.

This treatment was repeated several times.   The syrup was taken up in chloroform and dried with Na_2SO_4.   Most of the chloroform was removed in an air current and the final traces in a $CaCl_2$ desiccator at the water and oil pumps respectively.   The product was a brown, hard glass which could be ground to a yellow powder. Yield, about 5 gm. of ash-free material, soluble in ordinary organic solvents and insoluble in water.   Melting point 45–49°.

$C_{26}H_{26}O_{10}$.   Calculated.   C 62.65, H 5.26, OC_2H_5 9.04, OH 3.40
       498   Found.   " 63.20, " 5.43,   "   9.08, " 3.65
$[\alpha]_D^{25} = -52.5°$ (2.09 per cent in absolute alcohol).   No mutarotation

Oxidation of Glucose Cycloacetoacetic Ester with Perbenzoic Acid—100 gm. of glucose cycloacetoacetic ester and 200 gm. of $BaCO_3$ were suspended in 1000 cc. of water and cooled to 7°. 38 gm. of perbenzoic acid in 1180 cc. of ethyl acetate were added with rapid stirring.   The stirring was continued for 72 hours at room temperature until the perbenzoic acid had disappeared. The mixture was filtered, the aqueous layer separated, and most of the ethyl acetate removed from it by a current of air.   The water solution was concentrated at the pump at 50°, any precipitate being removed before the liquid became syrupy.   The syrup was taken up in 400 cc. of alcohol and the precipitate filtered off and washed with alcohol (150 cc.).   The alcoholic solution was placed in the ice box for several days and filtered.   The filtrate was concentrated to a heavy syrup under a vacuum at 46°. The syrup was extracted with 400 cc. of acetone and the precipitate filtered off and washed with acetone.   The acetone solution was concentrated under a vacuum (temperature 45°) to a syrup. The syrup was again taken up in acetone, a little norit added, and filtered.   The solution was concentrated to a syrup under a vacuum.   The syrup was taken up in chloroform and filtered. The chloroform was removed in a current of dry air, followed by evacuation at the water and oil pumps in a $CaCl_2$ desiccator. Yield, about 9 gm. of a brown, very hygroscopic glass, melting at 57–60°.   Ash content 3.62 per cent.

$C_{12}H_{18}O_8$.   Calculated.   C 49.65, H 6.25, 2OH 11.72, CH_3CO 14.83
       290   Found.   " 49.25, " 6.46,   " 11.20,   "   14.39

The analyses were calculated on an ash-free basis.

$$[\alpha]_D^{20} = -33.4° \text{ (2.22 per cent in water)}$$

The compound is definitely acidic in reaction. It possibly contains three hydroxyl groups, one of which fails to be acetylated under the conditions used. This is suggested by the observation that one hydroxyl of the isomeric compound was not benzoylated though it was acetylated.

SUMMARY

Two new highly unstable compounds of glucose and acetoacetic ester are described.

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MODIFICATIONS OF THE BIPYRIDINE METHOD FOR AVAILABLE IRON*

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Earlier work in this laboratory has shown that the efficiency of a food in regenerating hemoglobin in anemic rats is correlated, not with the total iron contained in the food, but rather with the inorganic, or available, iron content. Further work showed that the available iron could be determined by the use of α,α'-bipyridine (1–3). It was found, however, that for certain types of foods, namely leafy green materials and highly pigmented foods such as apricots, raisins, etc., the method described (1–3) could not be applied because of the presence of interfering pigments. We have reported the results of analyses on animal tissues in earlier papers. However, in many samples it is very difficult to estimate the iron because of turbidity. The purpose of this paper is to present modifications of the previous method, which widen its applicability and increase its accuracy, and to report the results obtained by the use of these methods.

EXPERIMENTAL

Modification of α,α'-Bipyridine Method for Pigmented Plant Tissues

The sample of material containing approximately 0.01 mg. of available iron was weighed directly into a 15 cc. centrifuge tube. 5 cc. of 10 per cent acetic acid, 1 cc. of a 0.2 per cent solution of α,α'-bipyridine in 10 per cent acetic acid, and about 0.25 gm. of hydroquinone were added. Hydroquinone has been substituted

for sodium hydrosulfite as the reducing agent because it is easier to purify. The hydroquinone was purified by dissolving in warm dilute hydrochloric acid and allowing it to crystallize from the cold solution. This process was repeated several times. The tube was then tightly stoppered and allowed to stand until maximum color had developed. 24 hours is usually long enough, but for some materials it is well to allow them to stand 4 or 5 days. Then, 5 cc. of a 7.5 per cent lead acetate solution were added and the tube again shaken and allowed to stand, this time overnight. The tube was then centrifuged and the color compared with that of a standard prepared with 0.01 mg. of Fe and made up to a volume of 11 cc. When fresh tissues are used, it is necessary to determine their water content and to take this into account in calculating the available iron.

In the case of very bulky materials, better results were obtained when the amounts of the reagents were doubled and 50 cc. tubes were used.

The use of lead acetate removed practically all of the chlorophyll and other pigments which would ordinarily interfere with the reading of the color. Best results were obtained when fresh, undried samples were used. Dried samples usually gave a brownish color which was not completely removed by lead acetate.

In order to check the accuracy of the modification, a number of materials which could be assayed by the unmodified method were analyzed by both the original and the lead acetate methods. The results are given in Table I, and it is evident that practically identical results were obtained in the case of Lima beans, navy beans, and almonds. Results for lettuce, parsley, spinach, grass, peas, raisins, apricots, and bananas obtained by this method are also included in Table I. Thus, this method is not only applicable to leafy green vegetables, but can be used for such foods as peas, which contain chlorophyll, and raisins and apricots, which contain other pigments.

Modification of α,α'-Bipyridine Method for Fresh Animal Tissues

The sample of fresh tissue, having been ground in a mortar, was weighed into a 15 cc. centrifuge tube. 5 cc. of 2.5 per cent trichloroacetic acid, 0.25 gm. of hydroquinone, and 1 cc. of a 0.2 per cent solution of α,α'-bipyridine in 10 per cent acetic acid were

TABLE I

Available Iron in Some Foods

Food	Total Fe per gm. dry material	Available Fe per gm. dry material	Per cent available	Method	
				Extractant	Precipitant
	mg.	*mg.*			
Almonds	0.042	0.037	88	10 % acetic acid	7.5% lead acetate
"	0.042	0.035	83	10 % " "	80 % alcohol
Lima beans	0.074	0.054	73	10 % " "	7.5% lead acetate
" "	0.074	0.052	70	10 % " "	80 % alcohol
Navy "	0.080	0.050	62	10 % " "	7.5% lead acetate
" "	0.080	0.048	60	10 % " "	80 % alcohol
Peas (canned)	0.122	0.110	90	10 % " "	7.5% lead acetate
" (fresh)	0.118	0.085	72	10 % " "	7.5% " "
Bananas	0.014	0.0086	61	10 % " "	7.5% " "
Raisins (Muscat)	0.064	0.040	62	10 % " "	7.5% " "
Apricots	0.043	0.021	50	10 % " "	7.5% " "
Grass (fresh)	0.153	0.051	33	10 % " "	7.5% " "
Raisins (bleached seedless)	0.051	0.015	30	10 % " "	7.5% " "
Lettuce (Batch 1)	0.230	0.066	28	10 % " "	7.5% " "
" (" 2)	0.304	0.064	21	10 % " "	7.5% " . "
Parsley (dried)	0.304	0.069	23	10 % " "	7.5% " "
Spinach (fresh)	0.352	0.067	19	10 % " "	7.5% " "
Beef liver (Batch 1)	0.260	0.180	69	2.5% CCl_3COOH	80 % alcohol
" " (" 3)	0.181	0.130	72	2.5% "	80 % "
" kidney	0.289	0.117	40	2.5% "	80 % "

added. The tube was tightly stoppered and the suspension shaken thoroughly and allowed to stand overnight. The next day 5 cc. of 80 per cent alcohol were added, the tube was again shaken, and allowed to stand overnight. The tube was then centrifuged and the color of the supernatant liquid compared with that of the standard. Here, again, it is necessary to determine the water content of the tissue and to include this factor in the calculation of available iron.

In the analyses of the tissues, the results of which are given in Table I, it was found that the resulting pH was well within the limits for maximum color formation, as given by Hill (4). However, in the analysis of tissues of unknown buffering action, it is advisable to determine the pH and to make certain that it is between 2.5 and 5 before color comparisons are made.

DISCUSSION

One or the other of the modifications of the bipyridine method described in this paper has been found to work for any sample of fresh food material. Some difficulties have been encountered in both plant and animal tissue after drying. However, if the sample has been dried very carefully, fairly accurate results may be obtained. It must be realized that drying may change the availability of the iron, so that the best procedure seems to be to analyze the fresh sample directly.

The determination of available iron in other leafy materials checks with our former results for spinach and alfalfa, and indicates that the per cent of available iron in this type of tissue is, in general, very low.

In the analysis of animal tissues the turbidity and dark color which interfered in the original method were found to be completely eliminated by the use of trichloroacetic acid, as described.

In our iron work we have found that when different samples of a foodstuff are used for analysis, the iron content may vary considerably. This is especially true in the case of liver. The iron content of liver depends upon a number of factors, including the age of the animal and the amount of copper and available iron in the ration of the animal previous to slaughter. Similarly, the iron content of plant tissues seems to be dependent upon the

amount of soluble iron in the soil upon which the plant has been grown.

SUMMARY

1. A modification of the α,α'-bipyridine method for available iron has been presented, which is applicable to pigmented plant tissues.

2. A modification of the same method has been presented which is applicable to fresh animal tissues.

3. The results of our analyses on various foods have been presented.

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THE CONFIGURATIONAL RELATIONSHIP OF METHYL-CYCLOHEXYLCARBINOL TO METHYLHEXYL-CARBINOL

By P. A. LEVENE and STANTON A. HARRIS

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The configurational relationship of the members of the homologous series of carbinols of the type $R \cdot CHOHC_6H_{11}$ (R = an alkyl) to those of the series $R \cdot CHOHC_6H_{13}$ has been formulated by Levene and Marker[1] by an indirect method. The configuration assigned to them on this basis was utilized later by Levene and Harris[2] for the formulation of the absolute configuration of carbinols of the type $R \cdot CH(OH)C_6H_5$.

In order to insure greater validity to the above two formulations, it seemed expedient to correlate the configurations of the carbinols of the cyclohexyl series with those of the *n*-hexyl series by direct chemical means. This has now been accomplished. The procedure was analogous to that employed by Levene and Harris[2] for the correlation of the hydrocarbons of the cyclohexyl series to those of the normal hexyl series. The individual steps in the reactions are given in the accompanying formulæ.

The rotation and the properties of the ether were compared with those of an authentic sample of the substance prepared from methylcyclohexylcarbinol.

Thus the configuration of dextro-methylcyclohexylcarbinol has been correlated to that of dextro-methyl-*n*-hexylcarbinol by direct chemical methods. The same configuration was formerly assigned to the dextro-methylcyclohexylcarbinol on the basis of theoretical considerations.

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).

[2] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **112**, 195 (1935).

$$CH_3$$
$$H—C—OH$$
$$COOC_2H_4$$

$\longrightarrow$

$$CH_3$$
$$H—C—OCH_3 \quad + \quad BrMgCH_2(CH_2)_3CH_2MgBr \quad \longrightarrow$$
$$COOC_2H_5$$

$[\alpha]_D^{23} = -3.93°$ $\qquad$ $[\alpha]_D^{23} = -30.10°$

$$CH_3$$
$$H—C—OCH_3$$
$$CH_2—CH_2$$
$$HOC \qquad CH_2$$
$$CH_2—CH_2$$

$\xrightarrow[CS_2]{K}$

$$CH_3$$
$$H—C—OCH_3$$
$$\overset{O}{\underset{\|}{KS—S—O—C}} \qquad \begin{matrix}CH_2—CH_2 \\ \qquad CH_2 \\ CH_2—CH_2\end{matrix}$$

Heat —

$$CH_3$$
$$H—C—OCH_3$$
$$C_6H_9$$

$\xrightarrow{H_2}$

$$CH_3$$
$$H—C—OCH_3$$
$$\begin{matrix}CH_2—CH_2 \\ HC \qquad CH_2 \\ CH_2—CH_2\end{matrix}$$

$[\alpha]_D^{23} = -12.43°$ $\qquad$ $[\alpha]_D^{23} = +2.93°$
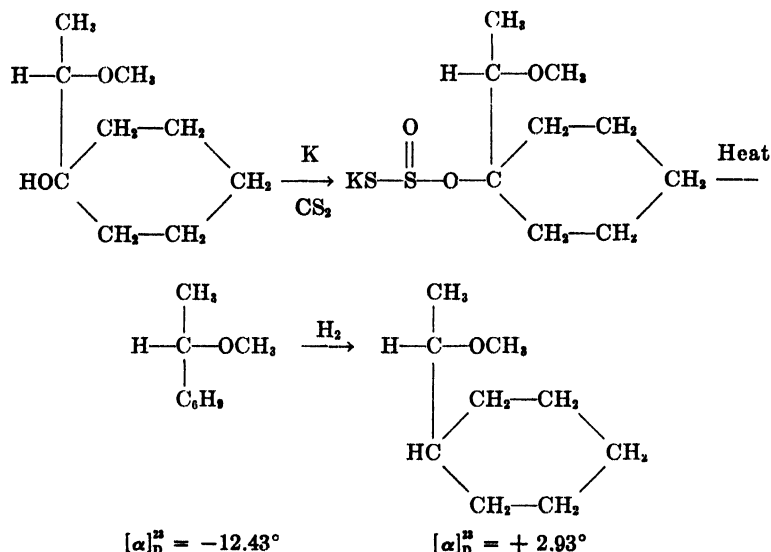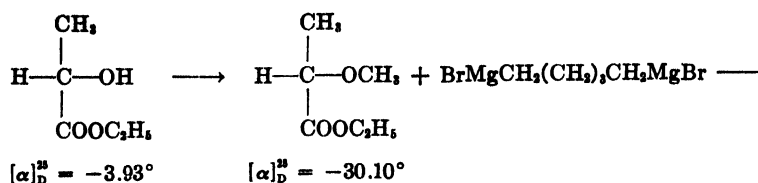
EXPERIMENTAL

Reaction between Ethyl Methyl Lactate and Pentamethylene-Di-magnesium Bromide—Commercial ethyl lactate having

$$[\alpha]_D^{25} = \frac{-8.10°}{2 \times 1.030} = -3.93°$$

was methylated with silver oxide and methyl iodide according to the method of Purdie and Irvine.[3] It was remethylated without any visible sign of further reaction. The product had

$$[\alpha]_D^{23} = \frac{-28.73°}{1 \times 0.955} = -30.10°$$

[3] Purdie, T. P., and Irvine, J. C., *J. Chem. Soc.*, **75**, 486 (1899).

42.0 gm. (0.4 mole) of this ester were added to an excess of penta-methylene-dimagnesium bromide cooled in an ice-salt bath. The reaction product was then hydrolyzed by being poured into an aqueous ammonium chloride solution. The carbinol was extracted with ether, washed with water, dried, and distilled. The yield of the fraction boiling at 99–104° at 22 mm. was 27 gm. (43 per cent of the theoretical). $d_4^{20.5} = 0.966$ (*in vacuo*). $n_D^{25} = 1.4544$.

$$[\alpha]_D^{25.5} = \frac{+7.39°}{1 \times 0.966} = +7.65° \quad .$$

4.412 mg. substance: 11.190 mg. CO_2 and 4.505 mg. H_2O
10.790 "   "   : 15.390 " AgI

$C_9H_{18}O_2$.	Calculated.	C 68.3, H 11.5, CH_3O 19.6
	Found.	" 69.2, " 11.4, " ·18.9

$$OCH_3$$
$$|$$
2-Methoxyethylcyclohexene-1 $\quad CH_3—CH—C_6H_9$

The above carbinol was distilled at atmospheric pressure at about 205° without any apparent decomposition. When distilled with potassium hydrogen sulfate, no product with a definite boiling point could be isolated. The method of Tschugaeff,[4] as used by Stevens,[5] was then resorted to. 23 gm. of the carbinol were dissolved in 300 cc. of dry ether and treated with an excess of metallic potassium which reacted vigorously, giving a red color. After standing overnight the solution was decanted from the potassium and treated with 2 moles of carbon disulfide. A solid mass was precipitated. After standing for 2 to 3 hours, 2.5 equivalents of methyl iodide were added and the solution was refluxed for 6 hours.

The potassium iodide was now filtered off and washed with dry ether and the ether solution was evaporated to a sirup which was transferred to a distilling flask and distilled over a free flame. Distillation commenced at 170° and the temperature rose to 200°. The fraction boiling at 170–180° was not free from mercaptan after being distilled twice from sodium. It was therefore extracted with mercuric chloride solution to remove the mercaptan and then

[4] Tschugaeff, L., *Ber. chem. Ges.*, **32**, 3332 (1899).
[5] Stevens, P. G., *J. Am. Chem. Soc.*, **54**, 3732 (1932).

with sodium carbonate solution. It was dried over anhydrous potassium carbonate and distilled. B.p. 168–171°. d_4^{23} = 0.883 (*in vacuo*). n_D^{25} = 1.4522.

$$[\alpha]_D^{23} = \frac{-10.98°}{1 \times 0.883} = -12.43°$$

4.600 mg. substance: 12.995 mg. CO_2 and 4.730 mg. H_2O
4.960 "   "   : 8.385 " AgI
$C_9H_{16}O$. Calculated. C 77.1, H 11.5, CH_3O 22.1
                Found.   " 77.0, " 11.5, "   22.3

$$\overset{\displaystyle OCH_3}{\underset{\displaystyle CH_3—CH—C_6H_{11}}{\vert}}$$

2-Methoxyethylcyclohexane

The unsaturated ether could not be reduced in pentane, but it was readily reduced in glacial acetic acid with Adams' catalyst and hydrogen in less than 1 minute. The resulting solution was poured into several volumes of water and extracted thoroughly with pentane. The pentane extract was washed with dilute sodium carbonate solution, dried over anhydrous potassium carbonate, and distilled. B.p. 168–171°. n_D^{25} = 1.4385. d_4^{23} = 0.860 (*in vacuo*).

$$[\alpha]_D^{23} = \frac{+2.52°}{1 \times 0.860} = +2.93°$$

3.245 mg. substance: 9.045 mg. CO_2 and 3.730 mg. H_2O
6.650 "   "   : 10.970 " AgI
$C_9H_{18}O$. Calculated. C 76.0, H 12.8, CH_3O 21.8
                Found.   " 76.0, " 12.9, "   21.8

Synthetic 2-Methoxyethylcyclohexane—The methylcyclohexylcarbinol was made by the reaction between cyclohexylmagnesium bromide and acetaldehyde, as used by Domleo and Kenyon,[6] a 61 per cent yield being obtained. The carbinol was converted to the half phthalate and resolved as the brucine salt. The recovered carbinol had a specific rotation of

$$[\alpha]_D^{15} = \frac{+4.99°}{1 \times 0.9^{nac}} = +5.50°; \ n_D^{25} = 1.4649$$

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[6] Domleo, A., and Kenyon, J., *J. Chem. Soc.*, 1841 (1926).

25 gm. of the active carbinol were treated with an excess of potassium in ether solution.   After several hours the reaction had moderated, so an excess of methyl iodide was added and the solution was refluxed for 6 hours.   After filtering and distilling, it was found that there still remained a considerable amount of carbinol. The methylation was repeated by refluxing the carbinol-ether solution in the presence of an excess of potassium metal.

The resulting product was distilled twice from sodium but was found to have a low methoxyl value, so it was·refluxed over potassium and then distilled.   This distillate was treated with an excess of phthalic anhydride in pyridine solution to form the phthalate.   After the pyridine had been removed by shaking with acid, the phthalate was extracted with 10 per cent sodium hydroxide solution.   The remaining chloroform solution was then dried and distilled.   B.p. 170–171°. $n_D^{25} = 1.4398$. $d_4^{23} = 0.867$ (*in   vacuo*).

$$[\alpha]_D^{23} = \frac{+6.85°}{1 \times 0.867} = +7.90°$$

4.516 mg. substance: 12.584 mg. CO_2 and 5.160 mg. H_2O
4.134 "   "   : 6.850 " AgI

$C_9H_{18}O$.	Calculated.	C 76.0, H 12.8, CH_3O 21.8
	Found.	" 76.0, " 12.8, " 21.9

HALIDE DISTRIBUTION IN BODY FLUIDS IN CHRONIC BROMIDE INTOXICATION*

BY MORTON F. MASON

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The metabolism of ingested bromide-containing compounds has received the attention of a good many investigators since Nencki and Schoumow-Simanowski (1) first showed the essential physical action of bromide to be that of replacing the chloride of the organism. The quantitative application of the Gibbs-Donnan membrane equilibrium theory to blood and extracellular fluids by Van Slyke, Wu, and McLean (2) later led to an examination of the behavior of the bromide ion (3) and the demonstration of its anomalous distribution between serum and erythrocytes (4, 5). Ratios of distribution of bromide between cells and serum, $(Br)_{cells}:(Br)_{serum}$ (expressed in mM per kilo of serum and cell water), were occasionally found to be above unity, and generally well above the accepted average chloride distribution of 0.67. Most of these observations were made either after intravenous injection of bromide salts or shortly after the taking of a large dose of bromide by mouth. Very few observations seem to have been made following prolonged bromide administration. Palmer and Clarke (6) have denied that the cell preferentially takes up bromide if sufficient time is allowed for equilibrium to be established.

The ratio of distribution of bromide between serum and spinal fluid, $(Br)_{serum}:(Br)_{spinal\ fluid}$, has been reported to be of the order of 1.5 to 2.0 (5, 7) (electrometric methods) and 2.4 to 2.8 (colorimetric methods) (8) in contrast to the average value of 0.89 for

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chloride calculated from the data of Fremont-Smith *et al.* (9) or to the value of 0.95 obtained by applying the Gibbs-Donnan law. Failure of the bromide to meet the calculated distribution ratio is not at all surprising for it is now recognized that spinal fluid is secreted and is not in equilibrium with serum; however, it is interesting that the ratio seems to differ so greatly from that for chloride.

Studies of urinary excretion of bromide have yielded conflicting results. Von Wyss' theory that the kidney does not distinguish between bromide and chloride has been confirmed (10–12) and denied (6, 13, 14).

Little is known about the distribution of chloride and bromide in some of the other secretions such as gastric juice and saliva in relation to that found simultaneously in the serum.

The purpose of this investigation was to study the distribution of bromide and chloride in the chronically intoxicated subject; *i.e.*, one who had been receiving bromide over a period of several weeks, and who presented clinical symptoms of bromide poisoning. Under these conditions simultaneous observations on the distribution of the halides between serum and cells, spinal fluid, urine, saliva, and gastric juice have been made. Some of the observations were made on hospital patients and the remainder were made on dogs.

Methods

Dogs were kept in metabolism cages and maintained on a standard ration (Bal Ra)[1] to which sodium bromide was added in amounts sufficient to make the daily intake 3 to 5 gm. The amount eaten each day contained 4.5 to 6 gm. of sodium chloride. Water intake was not restricted. After about 10 days definite symptoms of intoxication developed, including ataxia and apparent disorientation. The bromide intake was then adjusted so that the animals were maintained in an ataxic state but were still able to eat and take care of themselves in the cages. The level of the serum bromide was occasionally checked colorimetrically (modification of Wuth's method (15)) for it was found advantageous not to push the replacement of chloride by bromide above about 50 per cent, because of the resulting inanition and suscep-

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[1] Valentine Meat Juice Company, Richmond, Virginia.

tibility to respiratory infection when the animals were kept intoxicated over prolonged periods. Samples of blood and secretions for distribution measurements were taken after a 12 to 18 hour fast and 48 hours after the last bromide administration.

Patients were kept in the hospital 12 to 18 hours, fasting and untreated, before undergoing the sampling routine. Neither dogs nor patients were deprived of water during the fasting period.

Collection of Samples

Dogs—Amytal anesthesia was employed. A stomach tube was passed and the fasting contents aspirated and discarded. Stenson's duct was cannulated and the bladder catheterized and emptied. A few minutes were allowed for some urine to pass through the catheter. 3 to 5 mg. of pilocarpine were then administered intraperitoneally and a cisternal tap was made. A venous blood sample was taken anaerobically without stasis and a few minutes later the accumulated gastric juice was aspirated. Meanwhile the saliva from Stenson's duct was collected.

Patients—Gastric juice was obtained by means of a Rehfuss tube. The passage of the tube usually produced sufficient salivation for collection of a sample of mixed saliva; otherwise paraffin was chewed as a stimulant. The bladder was emptied about 40 minutes before the experiment (males) or catheterized as soon as the stomach tube was passed (females). Ergamine phosphate was given subcutaneously. A lumbar tap was made and then urine was collected voluntarily (males) or by catheter (females). Venous blood was taken anaerobically without stasis and the accumulated gastric juice aspirated.

The blood was defibrinated anaerobically with mercury (16), and the serum and cells carefully separated after centrifuging under oil. The secretions were centrifuged and the particle-free supernatant fluids were employed for analysis.

Analytical Methods—Moisture was determined by drying the fluids to constant weight at 105°. Total halide was determined on the dried samples after digestion with alkali as recommended by Sunderman (17), this value being used as a check on the separate electrometric determinations of chloride and bromide. To determine bromide and chloride 2 or more gm. of the fluid were weighed into a Coors crucible followed by about 0.2 cc. of 50 per

cent KOH.   After being dried and charred with a Bunsen burner the crucible was ashed in a muffle at 475–500°.   It was occasionally necessary to break up the carbonaceous residue, to add a little water or alcohol, and after drying, to return the crucible to the muffle a second time.   The ash was taken up in dilute nitric acid in sufficient amount to render the solution acid to methyl orange, then made up to a definite volume and filtered.   An aliquot of this was titrated electrometrically with the silver bromide electrode proposed by Hastings and van Dyke (3).   A saturated calomel electrode was connected to the titrating vessel by means of an agar-potassium chloride bridge in series with an agar-potassium nitrate bridge.   The bridges were changed frequently in order to prevent nitrate from diffusing back into the calomel half-cell.   Potentials were measured with a Leeds and Northrup acidity meter with an accuracy of 0.5 millivolt.   The aliquot was diluted to about 40 cc. with water, and titrated with 0.01 N silver nitrate admitted under the surface through a fine capillary tip at a rate not exceeding 0.2 cc. per minute.   The vessel was protected from direct light, and rapid mechanical stirring was employed throughout the titration.   In the neighborhood of the end-points the silver nitrate was added in 0.02 cc. increments, equilibrium often being attained only after about 5 minutes.   The end-point was obtained by geometrical analysis of the resulting curve.   In a good many instances only bromide was electrometrically titrated and chloride was calculated from the total halide determination.

The solubility products of silver bromide and chloride are sufficiently removed from one another to permit reasonable recovery of the bromide when its concentration is greater than 5 or 10 per cent of the chloride present.   The correction (3) for silver chloride precipitated with bromide established for blood filtrates (5, 13) was not found necessary for the ash solutions.

Results

Dogs—In Table I a series of observations on dogs is summarized.   The percentage replacement of chloride by bromide is calculated from the values for (Br)/(Br + Cl) in the fluid concerned.

. Table II gives the distribution ratios between serum and cells and serum and spinal fluid calculated from these figures.

TABLE I

Summary of Halide Distribution in Blood, Spinal Fluid, Urine, Gastric Juice, and Saliva of Dogs Intoxicated with Sodium Bromide

The results are expressed as milli-equivalents per kilo of water.

Dog No.	Date	Hema-tocrit	Serum				Cells				Spinal fluid			
			Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*
1	Aug. 9	30.0	122.8	53.5	69.3	43.6	85.2	38.4	46.8	45.2	133.1	41.4	91.7	31.1
1	" 24	29.7	123.5	59.3	64.2	48.0	87.1	45.1	42.0	51.8	126.8	42.2	84.6	33.2
2	Oct. 5	49.8	114.2	54.7	59.5	47.8	81.2	40.1	41.1	49.4	130.0	51.2	78.9	39.3
2	" 28	42.1	120.9	57.6	64.3	47.3	96.6	46.8	49.8	48.5	131.0	52.6	78.4	40.2
3	Nov. 12	40.9	120.9	65.2	55.7	53.9	88.6	48.7	39.9	55.0				

Dog No.	Date	Urine				Gastric juice				Saliva			
		Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*
1	Aug. 9	66.9	22.8	44.1	33.1	149.6	66.1	83.5	44.2	48.8	20.0	28.8	41.0
1	" 24	110.1	37.7	72.4	34.2	146.2	71.7	74.5	49.1	115.8	58.1	57.7	50.2
2	Oct. 5	73.2	32.3	40.9	44.2	143.2	64.5	78.7	45.0	90.0	38.6	51.4	42.9
2	" 28	93.9	33.7	60.2	35.9	148.0	75.0	73.0	50.7	67.4	31.5	35.9	46.8
3	Nov. 12	208.5	76.9	131.6	36.7	144.7	74.0	70.7	51.2	48.0	22.7	25.3	47.4

* R = per cent replacement of chloride by bromide ((Br) × 100)/(Br + Cl).

Patients—In Tables II and III the data obtained from patients are summarized in a similar manner.

In the patient E. W. observations were continued during a period in which the patient received 12 gm. of sodium chloride daily in addition to the amount (about 10 gm.) contained in the house diet. After 14 days the added salt was discontinued and 6 gm. of sodium bromide were given daily in its place. After 16

TABLE II

Halide Distribution Ratios between Cells and Serum and Serum and Spinal Fluid of Dogs and Patients

The results are expressed as milli-equivalents per kilo of water.

	Date	Cells:serum			Serum:spinal fluid			
		$\frac{(Br)_c}{(Br)_s}$	$\frac{(Cl)_c}{(Cl)_s}$	$\frac{(Br+Cl)_c}{(Br+Cl)_s}$	$\frac{(Br)_s}{(Br)_{sp.f.}}$	$\frac{(Cl)_s}{(Cl)_{sp.f.}}$	$\frac{(Br+Cl)_s}{(Br+Cl)_{sp.f.}}$	$\frac{R_u}{R_s}$*
Dog 1	Aug. 9	0.72	0.68	0.69				0.76
" 1	" 24	0.76	0.66	0.70	1.42	0.70	0.93	0.71
" 2	Oct. 5	0.73	0.69	0.71	1.30	0.70	0.90	0.92
" 2	" 28	0.81	0.77	0.80	1.13	0.81	0.93	0.76
" 3	Nov. 12	0.75	0.72	0.73	1.24	0.71	0.92	0.68
Patient B.R.	Dec. 21	0.80	0.79	0.79	1.20	0.77	0.94	0.86
" " †	" 29	0.85	0.77	0.78	1.73	0.87	0.91	0.84
" R.S.	Feb. 23	0.83	0.71	0.74	1.56	0.80	0.90	0.90
" V.D.	" 25	0.73	0.72	0.73	1.61	0.75	0.88	0.90
" W.A.	July 11	0.77	0.67	0.68	1.56	0.84	0.87	0.98

* R = per cent replacement of chloride by bromide $((Br) \times 100)/(Br + Cl)$. $R_u/R_s = R_{urine}/R_{serum}$.

† After 7 days treatment with sodium chloride added to the diet.

days the sodium bromide was discontinued and the patient was again given 12 gm. of sodium chloride daily. In this patient the gastric secretion and saliva were not studied, but some fifteen observations were made on the halide distribution in the blood, urine, and spinal fluid. Table IV includes the data obtained upon admission, after the period of sodium chloride therapy, after a period of sodium bromide administration, and again after a period of sodium chloride therapy.

TABLE III

Summary of Halide Distribution in Blood, Spinal Fluid, Urine, Gastric Juice, and Saliva of Patients with Bromide Intoxication

The results are expressed as milli-equivalents per kilo of water.

Patient	Date	Hema-tocrit	Serum				Cells				Spinal fluid			
			Total halide	Br	Cl	Per cent R^*	Total halide	Br	Cl	Per cent R^*	Total halide	Br	Cl	Per cent R^*
B.R.	Dec. 21	49.9	111.3	56.2	55.1	50.5	88.1	44.8	43.3	50.8	118.7	46.7	72.0	39.3
" †	" 29	47.2	113.5	11.1	102.4	9.8	87.9	9.4	78.5	10.7	124.6	6.4	118.2	5.1
R.S.	Feb. 23	44.3	111.1	26.2	84.9	23.6	82.2	21.7	60.5	26.4	123.2	16.8	106.4	13.6
V.D.	" 25	50.6	110.1	28.6	81.5	25.9	80.4	20.9	59.5	26.1	125.9	17.8	108.1	14.2
W.A.	July 11	28.4	107.4	12.3	95.1	11.5	72.8	9.5	63.3	13.1	121.5	7.9	113.6	6.5

Patient	Date		Urine				Gastric juice				Saliva			
			Total halide	Br	Cl	Per cent R^*	Total halide	Br	Cl	Per cent R^*	Total halide	Br	Cl	Per cent R^*
B.R.	Dec. 21		166.5	72.4	94.1	43.5	88.2	38.6	49.6	43.8	70.9	45.4	25.5	64.0
" †	" 29		96.5	7.9	88.6	8.2	102.6	15.7	86.9	15.3	50.4	9.0	41.4	17.9
R.S.	Feb. 23		155.3	33.1	122.2	21.3	48.5	5.9	42.5	12.3	40.7	14.6	26.1	36.0
V.D.	" 25		96.9	22.5	74.4	23.3	129.9	35.0	94.9	27.0	58.3	18.8	39.5	32.3
W.A.	July 11		54.2	6.1	48.1	11.2	133.6	15.8	117.8	11.8	63.2	10.0	53.2	15.9

For meaning of R see Table

After 7 days treatment with   chloride added to   die

TABLE IV

Halide Distribution in Blood, Spinal Fluid, and Urine of Patient E.W. after Salt Therapy and Sodium Bromide Administration

The results are expressed as milli-equivalents per kilo of water.

Date	Period	Hematocrit	Serum				Cells				Spinal fluid				Urine			
			Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*	Total halide	Br	Cl	Per cent R*
Nov. 28	Admission	42.9	107.4	46.7	60.6	43.5	94.1	36.8	57.3	39.2					45.5	14.5	31.0	31.9
Dec. 12	After 14 days 12 gm. NaCl added per day	42.2	112.8	7.6	105.2	6.7	80.3	5.5	74.9	6.8					101.6	5.3	96.3	5.2
" 30	After 16 days 6 gm. NaBr per day	43.3	110.6	35.8	74.8	32.4	83.3	28.0	55.3	33.6	124.3	22.4	101.9	18.1	81.2	17.5	63.7	21.5
Jan. 8	After 9 days 12 gm. NaCl per day	41.0	109.2	11.7	97.5	10.7	82.1	11.3	70.8	13.8	127.4	4.8	122.6	3.8	162.3	13.0	149.3	8.0

Distribution Ratios

Date	$\dfrac{(Br)_c}{(Br)_s}$	$\dfrac{(Cl)_c}{(Cl)_s}$	$\dfrac{(Br+Cl)_c}{(Br+Cl)_s}$	$\dfrac{(Br)_s}{(Br)_{sp.f.}}$	$\dfrac{(Cl)_s}{(Cl)_{sp.f.}}$	$\dfrac{(Br+Cl)_s}{(Br+Cl)_{sp.f.}}$	$\dfrac{R_{ut}}{R_s}$
Nov. 28	0.79	0.95	0.88				0.73
Dec. 12	0.72	0.71	0.71				0.78
" 30	0.78	0.74	0.75	1.60	0.73	0.89	0.67
Jan. 8	0.96	0.73	0.75	2.44	0.79	0.86	0.75

* See Table I for meaning.
† See Table II for meaning.

Halide Distribution between Serum and Cells—The data in Tables I to IV show that in chronic bromide intoxication the distribution ratio tends to be slightly greater for bromide than for chloride; that is, there is a relatively greater replacement of chloride by bromide in the cell than in the serum bathing it. The difference is small and variable, but is of the order reported for the serum-cell system after *in vitro* equilibration with sodium bromide (3). This is in contrast to the distributions found shortly after a large single oral or intravenous dose of sodium bromide, when bromide ratios as high as 2.0 have been observed (4). With time these ratios tended to fall towards the average value of 0.75, observed *in vitro*. The chloride ratios were reciprocally altered. In this investigation the chloride distribution ratios varied considerably in the patients, although they were fairly uniform in the dogs. They were not lowest in those instances when the bromide ratio was highest, which suggests that the extra bromide in the cell did not displace its equivalent of chloride. The ratios for total halide were elevated above the average normal range for chloride of 0.67 to 0.70, varying more in patients than in dogs. This was true in a number of single observations on both patients and dogs not reported here. It is doubtful if this can be due to differences in pH and degree of oxygenation in view of the method of collecting samples. The observation on patient E. W. on admission revealed a chloride ratio larger than the bromide ratio. The high value for the total cell halide and the fact that all later observations on this patient yielded higher bromide ratios suggest an analytical error rather than an anomalous distribution in this instance. Palmer and Clarke (6) deny that bromide replaces chloride to a greater extent in cells than in serum; however, they analyzed plasma and whole blood and calculated the cell halides, a procedure by which small differences may be masked.

Halide Distribution between Serum and Spinal Fluid—The halide distribution between serum and spinal fluid was determined in four instances in dogs and in five patients. In the case of patient E. W. nine observations were made during periods of salt therapy and bromide administration of which two are reported here. In all instances the distribution ratio for bromide was higher than

that for chloride; that is, relatively less bromide was found in the spinal fluid. The ratios observed were about half the values recently obtained by use of colorimetric methods (8), and somewhat lower than those obtained by electrometric methods after brief periods of bromide administration (7). Salt therapy tends to increase the ratio. Some indication of this is found in the values presented on patients E. W. and B. R. In the series of observations made on patient E. W. values as high as 4.5 were obtained during the periods of salt therapy. Apparently in prolonged intoxication a larger proportion of bromide enters the spinal fluid than after a single dose or after a brief period of administration of bromide salts. The high ratios observed in patient E. W. during salt therapy point to the fact that bromide leaves the spinal fluid more rapidly than the serum. It is difficult to explain Frey's finding (18) of equal bromide and chloride distribution in the serum and spinal fluid of dogs and cats.

In agreement with other observers, the distribution ratios for total halide were reasonably close to the average value of 0.89 calculated from the data of Fremont-Smith *et al.* The chloride ratios were lowered as a result of the unequal distribution of bromide.

Halide Distribution between Serum and Urine—Inasmuch as the concentration of halides excreted in the urine is a function of the rate of urine flow, it is simpler to use the term percentage replacement, $((Br) \times 100)/(Br + Cl) = R$, rather than the absolute concentrations of bromide and chloride. If the kidney did not differentiate between bromide and chloride, as has been claimed (10, 12), the replacements in the urine and serum would be equal in simultaneously taken samples, that is R_{urine}/R_{serum} would equal unity. In the case of dogs the value of this ratio of replacements except for one instance was about 0.7 (Table II). In the case of patients, the ratios were higher and more variable, not only in the cases reported here, but in several other isolated observations. Patient E. W., however, on whom this ratio was determined twelve times, constantly showed values between 0.67 and 0.79 with one exception when the ratio was 0.59. Salt therapy had no appreciable influence on this ratio of replacements. One patient, W. A., showed a ratio of 0.98, a value which was confirmed by another observation. It is of interest that this patient, whose kidneys

apparently behaved indiscriminately toward the two halides, had a mild sulfhemoglobinemia and a history of recent self-medication with acetanilide. These values for the ratio of replacements are considerably higher than those of Palmer and Clarke who reported for dogs an average value of about 0.4, this value rising when sodium chloride was administered. They reported one observation upon an untreated patient in whom the ratio of replacements was found to be 0.67. After 10 days on a high salt diet the value rose to 0.74. As stated, a high sodium chloride intake had no marked influence on the ratio of replacements of patient E. W. Two observations on dogs given caffeine followed by 250 cc. of 5 per cent glucose intravenously indicated that the resultant diuresis did not materially alter the ratio of replacements. This is in accord with Palmer and Clarke who state that urine volume changes are without effect. The data indicate that for patients, at least, the value of the ratio of replacements varies with individuals but is nearly constant for a given individual. This individual variation might well have some bearing on the clinical observation that the extent of bromide medication necessary to produce symptoms of bromism differs with individuals far more than one would expect from differences in sodium chloride intake. It is, of course, well known that the level of the serum bromide at which symptoms of intoxication are manifest is extremely variable.

Halide Distribution in Gastric Juice and Saliva—In these secretions as in urine the percentage replacement of chloride by bromide has been compared with that simultaneously existing in the serum. Distribution ratios such as those calculated for the serum-cell system have no significance. Tables I and III give the data obtained on dogs and patients and the calculated replacements. In dogs the replacement of bromide by chloride in the gastric juice approximated that found in the serum, being slightly larger in two instances and slightly smaller in three instances. In patients there was a similar variation but greater in degree. The replacement in the parotid saliva of dogs was in four instances slightly smaller than that in the serum, and in one instance somewhat greater. The mixed saliva of patients, however, in each instance was found to have a considerably greater replacement of chloride by bromide than the serum. In view of the fact that

the replacement in dog parotid saliva is similar to that in serum, it is possible that one or more of the other glands in the patients secrete bromide with marked preference over chloride. The matter needs further investigation. The rapidity of excretion of one of the other halides, iodide, by the salivary route is well known.

It was felt that the demonstration of organic combinations of bromide in the fluids or tissues of either dogs or patients with bromism would be of aid in offering an explanation of at least some of the peculiarities of bromide distribution reported in this paper. An intensive search for such combinations in cells, serum, and saliva failed to reveal evidence for the existence of such compounds. Dialysis procedures and extractions with various organic solvents were employed. One of the methods of approach was quite similar to that used by Peters and Man (19) in their demonstration of the presence of lipoid-bound chlorine in patients with nephrosis. It would be of interest to use their exact technique, for as they pointed out, it must be followed closely in order to demonstrate successfully lipoid-bound chlorine.

SUMMARY

The distribution of bromide and chloride in simultaneously taken samples of blood, spinal fluid, urine, gastric juice, and saliva from dogs and patients with chronic bromism has been determined.

1. The distribution ratio for bromide between cells and serum, $(Br)_{cells}:(Br)_{serum}$, is slightly higher than that for chloride and is of the order of that found after *in vitro* equilibration of blood with sodium bromide. The high total halide ratios and absence of low chloride ratios suggest that the extra cell bromide has not displaced its equivalent of chloride.

2. The distribution ratio for bromide between serum and spinal fluid, $(Br)_{serum}:(Br)_{spinal\ fluid}$, is much greater than that for chloride, being of the order of 1.5 for patients and 1.2 for dogs. The distribution ratio for chloride is lowered, but that for total halide is equal to that found in normal individuals. Sodium chloride treatment increases the value of the bromide distribution ratio in patients.

3. The replacement of chloride by bromide in the urine of both dogs and patients is less than that found simultaneously in the serum. The value for the ratio of replacements, $R_{urine}:R_{serum}$,

was fairly constant for one individual for whom it was determined a number of times. In several patients and dogs it was found to be higher than reported by others. It was not materially affected by diuresis or changes in sodium chloride intake.

4. The replacement of chloride by bromide in the gastric juice of both patients and dogs was sometimes greater and sometimes smaller than that in the serum.

5. The replacement of chloride by bromide in dog parotid saliva was approximately equal to that existing in the serum; human mixed saliva, on the other hand, had a much greater replacement of chloride by bromide than the serum. One or more of the human salivary glands must preferentially secrete bromide to a marked degree.

6. No evidence was obtained for the existence of organic bromide combinations in cells, serum, or saliva.

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ON THE GLUCOSE METABOLISM OF TRYPANOSOMES (TRYPANOSOMA EQUIPERDUM AND TRYPANOSOMA LEWISI)

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Pathogenic as well as saprophytic trypanosomes utilize glucose and probably obtain from it the energy required for life and their especially active motion. It has been shown previously that glucose is decomposed by trypanosomes aerobically as well as anaerobically. Measurements on the oxygen consumption and acid production have been reported (1–4). Little is known, however, about the chemical mechanism of glucose decomposition.[1] The present paper reports experiments by which the main steps in the breakdown of glucose by *Trypanosoma equiperdum* and *Trypanosoma lewisi* have been established.

Experiments with Trypanosoma equiperdum

Manometric measurements of the oxygen consumption showed that the amount of oxygen consumed by a certain number of trypanosomes is rather variable. This is probably due to the fact that the oxygen consumption, even in well buffered solutions, is not proportional to the time, but decreases continuously. The rate of the metabolism, therefore, depends to a great extent on the time required for the preparation of the trypanosome suspension. (An example is given in Table I.)

The rate of the oxygen consumption depends also on the medium in which the trypanosomes were kept before and during the experiment. This is strikingly demonstrated in an experiment, the results of which are given in Table II. It shows the effect of cell-

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[1] A preliminary paper on some of the experiments with *Trypanosoma equiperdum* was published by Reiner and Smythe (5).

free peritoneal exudate of a guinea pig on the respiration of the trypanosomes. Thus, it was not possible to establish absolute values for the rate of the metabolism. It was necessary to com-

TABLE I

Influence of Number of Trypanosomes (Trypanosoma equiperdum), Time, and Medium on Oxygen Consumption

Gas, 100 per cent O_2; buffer, 0.1 M phosphate; initial pH, 7.6.

Medium...............	Buffer	2 parts buffer + 1 part serum	
No. of trypanosomes per cc...............	7.2×10^7	1.83×10^7	1.83×10^6
Time	O_2 used per 10^{10} trypanosomes per hr.		
min.	*cc.*	*cc.*	*cc.*
0– 15	23.5	44.7	95.5
15– 45	9.1	41.8	59.2
45– 90	5.28*	38.9	38.0
90–210		34.0	31.3
210–215		33.2†	28.0‡

* Interval 45 to 120 minutes.
† Movement slightly impaired.
‡ Movement strongly impaired.

TABLE II

Comparison of Effect of Glucose and Exudate Plus Glucose on Oxygen Consumption of Trypanosoma equiperdum

Gas, air; buffer, 0.1 M phosphate; initial pH, 7.6.

Buffer, *cc*...............	1.00	1.00	1.00
0.85% NaCl, *cc*.....	0.6	0.6	0.3
6% glucose, *cc*...........		0.2	0.2
Exudate, *cc*.............			0.3
Trypanosomes, *cc*.......	0.2	0.2	0.2
Time	O_2 consumption		
min.	*c.mm.*	*c.mm.*	*c.mm.*
20	1.4	3.6	69.6
45	1.4	9.5	168.0
70	1.4	16.1	248.5

pare analytical data obtained simultaneously on the same suspension of trypanosomes.

The carbon dioxide liberated from solutions containing bicar-

bonate was approximately proportional to the oxygen consumption. The ratio of carbon dioxide liberated from media containing bicarbonate (a measure of the total acid produced) to the oxygen consumed averaged 1.8 ± 0.15.

In another set of experiments the acids produced (except carbon dioxide) were determined by titration and compared with the glucose decomposed. The ratio of equivalents of acid to moles of glucose was 1.74 ± 0.20 in aerobic experiments and 0.92 ± 0.13 in anaerobic experiments.

These experiments suggest that 1 equivalent of acid is produced from 1 molecule of glucose anaerobically and that 2 equivalents of acid are produced from 1 molecule of glucose aerobically. 1 molecule of oxygen is required for the latter process, that is, for the oxidation of glucose.

• To ascertain this further, the rate of glucose decomposition was compared with the rate of oxygen consumption. It was found, as expected from the comparison of acid production and oxygen consumption, that for 1 molecule of glucose, 1 molecule of oxygen was used up during long time intervals. The glucose decomposition took place at a higher rate at the beginning of an experiment and decreased with time more rapidly than the oxygen consumption. Correspondingly, the ratio of oxygen consumed to glucose decomposed was small at the beginning of an experiment and was great at the end of an experiment (when almost all the glucose was used up).

These experiments suggest that glucose, even under aerobic conditions, is first broken down without oxidation by molecular oxygen and that an intermediate product, which is not an acid, is responsible for the oxygen consumption. The resulting product is an acid. (*Cf.* Table III.)

In further experiments attempts were made to determine the products resulting from the anaerobic and aerobic decomposition of glucose. Carbon dioxide was not produced under anaerobic conditions and only very small amounts of it were found under aerobic conditions. The R.Q. was less than 0.1; *i.e.*, 0.062 ± 0.004. The acid produced was not distillable by steam from solutions acidified with a small excess of sulfuric acid. Only a negligible fraction (2 to 12 per cent) of the acid was lactic acid.

A precipitate was obtained when the media in which the try-
panosomes had been kept under anaerobic conditions for several
hours at 37° were acidified with hydrochloric acid and mixed with
a concentrated solution of phenylhydrazine acetate.   This precipi-
tate consisted of yellow needles which were centrifuged and re-
crystallized from alcohol. They had a melting point of 183.2°.
The determination of the mixed melting point proved that the
precipitate was the phenylhydrazone of pyruvic acid.   The hy-
drazone was redissolved by adding sodium carbonate and re-
crystallized by acidification, washed, dried, and weighed.   It
corresponded to 50 to 75 per cent of the acid determined by
titration.

Pyruvic acid was also formed under aerobic conditions.   It was
stated above the total acid produced per mole of glucose was

TABLE III

Comparison of Rate of Glucose and Oxygen Consumption of Trypanosoma
equiperdum

Gas, air; medium, 0.1 M phosphate buffer; initial pH, 7.6.

Time	Glucose*	Oxygen	Oxygen:glucose
min.	*c.mm.*	*c.mm.*	
5– 35	167	140	0.84
35– 65	68.5	73.5	1.02
65–120	46.0	65.5	1.42
5–120	281.5	279	0.99

* 180 mg. of glucose = 22,412 c.mm.

roughly twice as much under aerobic conditions as under anaerobic
conditions.   The amount of pyruvic acid formed per mole of
glucose was also roughly twice as much under aerobic conditions
as under anaerobic conditions, the yield being in both cases 50 to
75 per cent of the total acid.   This indicated that the acid formed
aerobically was also mainly pyruvic acid.

Since it was established by these experiments that molecular
oxygen is not involved in the first step of glucose decomposition,
even under aerobic conditions, and that a resulting product is
oxidized with 1 molecule of oxygen per molecule of glucose (or
more exactly the products formed from 1 molecule of glucose),

and since it was established that the resulting acid is in both steps
of the reaction pyruvic acid, the path of the glucose decomposition
can be expressed by the following two equations.

(1) $\qquad C_6H_{12}O_6 \rightarrow CH_3COCOOH + (C_3H_8O_3)$
(2) $\qquad (C_3H_8O_3) + O_2 \rightarrow CH_3COCOOH + H_2O$
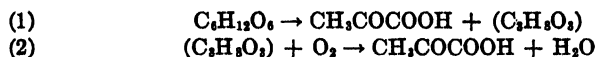
The formula in parentheses is the empirical formula of the anaero-
bic breakdown product.   It is the empirical formula of glycerol.

Kudicke and Evers (6) found that glycerol supports the life
of trypanosomes.   The substances, gluconic acid, methylglyoxal,
glyceric aldehyde, glyceric acid, pyruvic acid, lactic acid, glycerol,

TABLE IV

*Glucose Metabolism in Serum-Containing Medium (Trypanosoma
equiperdum)*

Medium, 1 part of rabbit serum + 2 parts of Ringer's solution.

	Glucose used	Acid formed*	Acid: glucose	Phenylhydrazone of pyruvic acid			Glycerol		
					Acid	Glucose		Acid	Glucose
	micro-moles	*micro-equiva-lents*		*mg.*	*mole per cent*	*mole per cent*	*mg.*	*mole per cent*	*mole per cent*
Anaerobic	782	770	0.98	68.0	50	49	17.2	24	24
"	428	452	1.05	51.0	64	67	52.4	126	132
Aerobic	1050	1690	1.61	185.2	62	99	Trace		
"	630	1300	2.06	145.6	63	129	None		

* Except CO_2.

glycerophosphoric acid, acetaldehyde, ethyl alcohol, glycol, and
formaldehyde, were tested concerning their ability to support the
life of trypanosomes; glycerol alone did so but only under aerobic
conditions.   Quantitative experiments with glycerol showed that
1 molecule of oxygen was necessary for the production of 1 mole-
cule of acid (liberation of carbon dioxide from solutions containing
an excess of bicarbonate).   The average ratio of acid produced
per molecule of oxygen consumed was 0.99 ± 0.03.   Less than 5
per cent of the acid was carbon dioxide (R.Q. less than 0.05 in
three experiments).   The acid produced was identified as pyruvic
acid.   The yields were somewhat higher than those obtained in
experiments with glucose.

The amount of glycerol produced from glucose under anaerobic conditions was estimated by the method of Zeisel and Fanto (7). It was found in accordance with Equation 2 that 1 molecule of glycerol is produced from 1 molecule of glucose under anaerobic conditions. 88 moles per cent was the average. No glycerol or only traces were found under aerobic conditions.

The glucose decomposition follows the same course if serum, peritoneal exudate, or other substances are present, which increase the life span of trypanosomes and assist oxygen consumption *in vitro* (*cf*. Table IV).

A comparison between normal trypanosomes and trypanosomes resistant to arsenicals indicated no qualitative difference in their carbohydrate metabolism. This part of the study is being continued.

Experiments with Trypanosoma lewisi

The oxygen consumption of these trypanosomes is proportional to the time throughout a fairly long interval. They are apparently less damaged under the conditions prevailing in *in vitro* experiments than *Trypanosoma equiperdum*, yet the rate of their metabolism, oxygen consumption, as well as glucose decomposition, is much less than that of *Trypanosoma equiperdum*. The initial rate of oxygen consumption for *Trypanosoma equiperdum* was about 50 cc. of oxygen per hour per 10^{10} trypanosomes.[2] The same value for *Trypanosoma lewisi* is about 5 cc. of oxygen (*cf*. Table V).

The ratio of oxygen consumed to carbon dioxide liberated from solutions containing bicarbonate is 2.38 ± 0.13.

Comparison of the acid (except CO_2) produced with the glucose consumed showed that under anaerobic, as well as under aerobic conditions, about 2.5 equivalents of acid were produced per molecule of glucose. The average of fourteen anaerobic experiments was 2.44 ± 0.36 and the average of twelve aerobic experiments was 2.44 ± 0.34. Thus, the acid production, if carbon dioxide is not included, is the same under aerobic and anaerobic conditions.

Comparison of the oxygen consumption with glucose decompo-

[2] This is approximately the number of trypanosomes present in a rat heavily infected with *Trypanosoma equiperdum* but more than the number usually attained in rats infected with *Trypanosoma lewisi*.

sition showed that less than 1 molecule of oxygen is used for 1 molecule of glucose decomposed. The ratio of oxygen to glucose increases with time and apparently approaches the value 1.0. These facts indicate that *Trypanosoma lewisi*, like *Trypanosoma equiperdum*, first decomposes glucose without oxidation by molecular oxygen even under aerobic conditions. A resulting product

TABLE V

Rate of Oxygen Consumption and Its Variation with Time (Trypanosoma lewisi)

Gas, air; medium, 0.1 M phosphate buffer; initial pH, 7.6.

Time	O_2 per 10^{10} trypanosomes per hr.
min.	*cc.*
0– 15	4.84
15– 30	4.68
30– 50	4.83
50–120	2.23

TABLE VI

Comparison of Rate of Glucose Decomposition with That of Oxygen Consumption and Carbon Dioxide Production (Trypanosoma lewisi)

Gas, air; medium, 0.1 M phosphate buffer; initial pH, 7.6.

Time	Glucose*	Oxygen	O_2:glucose	CO_2	CO_2:O_2
min.	*c.mm.*	*c.mm.*		*c.mm.*	
5– 45	241	115	0.48	115	1.00
45–120	141	155	1.10		
5–120	382	270	0.71	267	0.99

* 180 mg. of glucose = 22,412 c.mm.

is then oxidized. The oxidation of this product yields mainly carbon dioxide. The R.Q. approaches the value 1.0 (*cf.* Table VI).

Qualitative tests showed that the medium in which the trypanosomes were kept under anaerobic conditions for several hours did not contain lactic acid or any other hydroxy acids, nor did it contain keto acids, formic acid, carbon dioxide, or any substance which, like oxalic acid, would be rapidly oxidized by permanganate at 100° in acid solution. About 10 per cent or less of the

total acid formed was distillable from the aqueous solution by steam. The distillate contained acetic acid.

The non-volatile acid could be extracted with ethyl ether by continuous percolation of the concentrated and acidified solution used as medium. After evaporation of the ether, crystals were obtained in various preparations, which melted between 178–182°. Recrystallization from water yielded a product which melted at 185° and was identified as succinic acid by mixed melting point determination. The crystals obtained from the ether extract corresponded to 64 to 78 per cent (average 73 per cent) of the total acid titrated.

TABLE VII

Determination of Acetic Acid and Ethyl Alcohol

Medium, Ringer's solution.

	Glucose used	Total acid	Acetic acid	Equivalent of total	Uranyl acetate test	Ethyl alcohol	Iodoform test
	micromoles	*micro-equivalents*	*micromoles*	*per cent*		*micromoles*	
Anaerobic	1192	3346	250	7.5	+	182	+
"	1089	3382	247	7.3	+	97	+
Aerobic	367	1100	175	15.9			
	728	2000	394*	19.7	+	69	+

* Contains 16 microequivalents of formic acid.

Under aerobic conditions 15 to 25 per cent of the total acid was distillable by steam (average 22.3 per cent). Of this 2 to 4 per cent (less than 1 per cent of the total acid) was formic acid, the rest presumably being acetic acid (uranyl acetate test). The remaining (non-volatile) acid was again extracted with ether and identified as succinic acid. The yield in succinic acid thus isolated was 72 to 88 per cent (average 78.3 per cent) of the total non-volatile acid. The purity of these products, as determined by titration with NaOH, varied between 88 and 98 per cent. The melting point was between 177.8–180.6°.

The only non-acidic substance found under anaerobic and also under aerobic conditions was ethyl alcohol (iodoform test). Acetaldehyde and formaldehyde were not present. The yields in ethyl alcohol were small. They corresponded to 1 molecule of ethyl alcohol for 5 molecules of glucose or less. (*Cf.* Table VII.)

These results indicate that the first step in the glucose metabolism of *Trypanosoma lewisi* does not involve oxidation by molecular oxygen even under aerobic conditions. It consists in the formation of 1 molecule of succinic acid and 1 molecule of glycol (or acetaldehyde+H_2O) from 1 molecule of glucose.

(3) $C_6H_{12}O_6 \rightarrow COOHCH_2CH_2COOH + C_2H_6O_2$

This is followed under anaerobic conditions by the formation of acetic acid and ethyl alcohol, probably through formation of acetaldehyde and H_2O.

(4) $2C_2H_6O_2 \rightarrow 2CH_3COH + 2H_2O \rightarrow CH_3COOH + CH_3CH_2OH + H_2O$
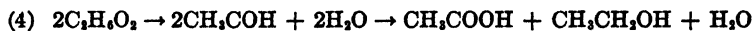
The reaction of Equation 4 also takes place under aerobic conditions, and in addition the following oxidations occur.

(5-a) $2CH_3COH + 4O_2 \rightarrow 2HCOOH + 2CO_2 + 2H_2O$
(5-b) $2HCOOH + O_2 \rightarrow 2CO_2 + 2H_2O$
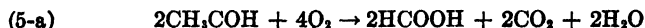
This scheme is in agreement with the results obtained. It accounts for the fact that the total acid, with the exception of CO_2, is approximately the same in aerobic and anaerobic experiments (Equations 3 and 4). It also accounts for the fact that oxygen is consumed only after the glucose decomposition is well started. The R.Q. should, however, be 0.8 according to Equations 5-a and 5-b. As a rule, values close to 1.0 were obtained (0.98±0.02). So far we are unable to account for this discrepancy. It is possible that part of the carboxylic acids is also oxidized or decarboxylated. The possibility that part of the glucose is completely oxidized also has to be considered. Whether or not it goes through the steps of succinic acid and acetaldehyde has not yet been determined. The yields in alcohol and acetic acid were much lower than that expected according to this scheme. 1 molecule of acetic acid and 1 molecule of ethyl alcohol should be formed from 2 molecules of glucose under anaerobic conditions (*i.e.*, 20 per cent of the total acid should be distillable by steam). Less than half of this amount was actually found. This indicates that the reaction of Equation 4 does not go to completion. The fact that the acid distillable by steam was consistently higher under

aerobic conditions than under anaerobic conditions suggests that the reaction of Equation 6 also takes place.

(6) $2CH_3COH + O_2 \rightarrow 2CH_3COOH$

It is also conceivable that an intermediate product oxidizes part of the formic acid formed to CO_2. This would account for the fact that the R.Q. values found were considerably higher than those calculated on the basis of Equations 5-a and 5-b.

DISCUSSION

Both pyruvic and succinic acid are often encountered as breakdown products of the glucose metabolism of bacteria and other cells. They have been considered mostly as intermediate products or side products. To our knowledge the case of *Trypanosoma lewisi* reported here is the first in which succinic acid was found to be the main end-product of uninfluenced anaerobic and aerobic glucose metabolism. Furthermore, the case of *Trypanosoma equiperdum* is the first in which the end-product of uninfluenced anaerobic glucose metabolism is pyruvic acid and glycerol and in which mainly pyruvic acid is formed under aerobic conditions.

It is of interest to discuss what, if any, the intermediate steps of the anaerobic metabolism might be in these cases, although little can be said with certainty about them for they are probably formed and decomposed intracellularly.

The anaerobic formation of glycerol and pyruvic acid may be a result of a chain of reactions which, except for the participation of phosphoric acid, which is questionable here, might be similar to that discussed by Meyerhof (8)[3] as a part of the glucose decomposition by muscle.

$$\text{Glucose} \rightarrow 2 \text{ triose} \rightarrow \genfrac{}{}{0pt}{}{\text{glycerol}}{\text{glyceric acid} \rightarrow \text{pyruvic acid}}$$
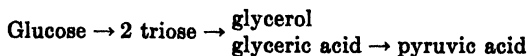
Phosphorylation was not ruled out here, but there was no evidence suggesting its occurrence. The fact that glyceric aldehyde did not support the life of trypanosomes does not necessarily contradict this scheme.

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[3] See this review for additional references.

In the case of succinic acid formation by *Bacillus coli*, Grey (9) suggested that it is formed through the oxidative condensation of 2 acetic acid molecules. De Graaff and Le Fèvre (10) suggested that succinic acid might be formed through condensation, decarboxylation, and oxidation from 2 pyruvic acid molecules. Both of these possibilities are improbable in light of the facts (1) that neither acetic nor pyruvic acid was attacked by *Trypanosoma lewisi* and (2) that oxidation through molecular O_2 was excluded and oxidation of acetic or pyruvic acid by an unknown H acceptor was unlikely. (The environment was strongly reducing as indicated by the negative potentials obtained with blank platinum electrodes and by the complete reduction of methylene blue.)

Our results seem to indicate that the mechanism of glucose decomposition is very different with *Trypanosoma equiperdum* and *Trypanosoma lewisi*. Surprising is the fact that while *Trypanosoma equiperdum* produces two 3-carbon atom compounds, *Trypanosoma lewisi* forms a 2- and a 4-carbon compound.

SUMMARY

1. *Trypanosoma equiperdum* decomposes glucose anaerobically by forming 1 molecule of glycerol and 1 molecule of pyruvic acid from 1 molecule of glucose. This reaction is also the first step in the glucose metabolism under aerobic conditions. It is followed by the oxidation of glycerol to pyruvic acid and water so that the oxidative decomposition of glucose yields 2 molecules of pyruvic acid per molecule of glucose. Comparatively small amounts of lactic acid and carbon dioxide were found, possibly due to the metabolism of contaminating cells (leucocytes). The presence of serum or plasma does not alter the course of glucose decomposition. It seems to increase the rate of the anaerobic reaction. The glucose metabolism of normal trypanosomes and trypanosomes resistant to arsenicals showed no qualitative differences.

2. *Trypanosoma lewisi* decomposes glucose anaerobically by forming 1 molecule of succinic acid and presumably 1 molecule of glycol. The glycol is decomposed further, presumably to acetaldehyde and water and the acetaldehyde undergoes dismutation so that acetic acid and ethyl alcohol are also formed. The same reactions take place under aerobic conditions but in addition

acetaldehyde is oxidized to formic acid, carbon dioxide, and water and most of the formic acid is further oxidized to carbon dioxide and water. The final products of the aerobic metabolism are therefore succinic, acetic, formic, and carbonic acids and ethyl alcohol.

No indications were found that phosphorylation is involved in the glucose metabolism of either species.

Methods

Trypanosomes—The trypanosome strains were laboratory strains. The *Trypanosoma equiperdum* strain was kept for 6 years in white rats and guinea pigs. The *Trypanosoma lewisi* strain was kept for 2 years in white rats. The trypanosomes were obtained from the rat blood by fractional centrifugation previously described (2). To separate *Trypanosoma lewisi* from the plasma it was necessary to centrifuge for 15 minutes at 2300 R.P.M.

Medium—The trypanosomes were suspended in Ringer's solution heavily buffered with sodium bicarbonate. The Ringer's solution was made up in a 10 times concentrated form, and without buffer. It contained 72 gm. of sodium chloride, 1.92 gm. of anhydrous calcium chloride, and 3.36 gm. of potassium chloride in a liter. This solution was diluted with water, the buffer used, and glucose. The final bicarbonate concentration was usually 0.025 to 0.050. Less bicarbonate was used in some of the manometric experiments. If only the oxygen consumption was measured, phosphate buffer (0.1 M, pH 7.6) was used. The solutions always contained 0.3 per cent glucose. In some instances 2 parts of Ringer's solution were diluted with 1 part of fresh rabbit serum (*cf.* Table IV). For the preparation of peritoneal exudate a guinea pig weighing about 500 gm. was injected with 20 cc. of phosphate buffer (0.1 M, pH 7.6) containing 0.3 per cent glucose. Exudate was taken through a glass cannula 4 hours later. It was centrifuged free from cells before use. All experiments were carried out at 37° under as nearly as possible sterile conditions.

Gasometric Measurements—The oxygen consumption, the carbon dioxide consumption, and the carbon dioxide liberated from bicarbonate were measured in the Warburg-Barcroft apparatus. The carbon dioxide production in a solution buffered with phos-

phate was measured as the difference of the gas expelled by dilute sulfuric acid from the medium containing the trypanosomes, at different time intervals. The carbon dioxide liberated from bicarbonate was determined by Warburg's 2 volume method based on the difference in the solubility of oxygen and carbon dioxide in water.

The acids produced were titrated with 0.02 N sodium hydroxide, with phenolphthalein as indicator. The centrifuged solution was first acidified with an excess of sulfuric acid, then boiled for several minutes.

The "volatile acid" was defined as the acid which could be distilled over by steam from a solution acidified with sulfuric acid. The solutions were aerated for 30 minutes before distillation to drive off carbon dioxide.

Pyruvic Acid—The medium was acidified, concentrated in a vacuum, and centrifuged free from insoluble material, then mixed with an excess of phenylhydrazine acetate. The precipitate was redissolved by adding sodium carbonate and recrystallized by acidification, centrifuged, washed, dried, and weighed.

Glucose—Hagedorn-Jensen method (11).

Lactic Acid—Friedemann, Cotonio, and Shaffer (12).

Glycerol—Zeisel and Fanto (7).

Succinic Acid—This was extracted with ether from solutions concentrated on the water bath. Continuous extraction (13) was used. The ether, acetic acid, and hydrochloric acid were evaporated. The crystals obtained were weighed and titrated with sodium hydroxide. The melting points taken on this material are uncorrected. The substance was recrystallized from water for identification by mixed melting point.

Formic Acid—This was determined by reduction of mercuric chloride (14).

Acetic Acid—This was determined by titration of the steam distillate. It was identified as uranyl acetate.

Acetaldehyde—The sulfite method and the fuchsin test were used on the distillate.

Ethyl Alcohol—The neutralized medium was fractionally distilled and the distillate oxidized with 0.1 N potassium dichromate in acid solution in sealed containers.

The experiments given in Tables I to VII are typical representatives of several experiments. Unless otherwise stated averages were calculated from the results of five or more experiments.

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THE DETERMINATION OF CAROTENE AND XANTHO-PHYLL BY A SINGLE DISTRIBUTION BETWEEN LIQUID PHASES

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The method described below may be applied to the analysis of mixtures of two substances which become differently distributed between immiscible solvents. It may be used when a characteristic, common to both substances, is easy to determine—such as the acid or base value, the oxidation or reduction value, or the color value. We have used the color value for the estimation of mixtures of carotene and xanthophyll when the quantity of either falls in the range from 0.2 to 5.0 micrograms.

When hexane and aqueous methyl or ethyl alcohol are shaken together, two phases form. If a mixture of carotene and xanthophyll is present in such a system, the upper phase will contain most of the carotene and the lower will contain much of the xanthophyll. The separation is never complete; but by repeated extraction of the phases with fresh solvents, used in suitable quantity, any desired degree of separation can be obtained. When only small quantities of carotenoids are present, such a procedure is liable to produce a dilution inconvenient for quantitative analysis; it is time-consuming and attended by inevitable loss. We have therefore undertaken the study of conditions which will permit the accurate colorimetric determination of carotene and xanthophyll in a mixture when a single distribution is made between a pair of suitable solvents.

Theory of Method

When a system of two liquid phases is formed at a specified temperature and pressure by bringing into equilibrium definite

quantities of hexane and alcohol and water, the volumes of the phases will be definitely fixed. If such a system contains a known quantity of a pigment, soluble in both phases, the concentration of the pigment in each phase will assume a definite value. From a determination of the concentration in either phase, it is therefore possible to estimate the total amount of the pigment present. The determination can be made most accurately with the spectrophotometer. For many purposes, the colorimeter suffices, a solution of the pigment in question being used as standard, or some arbitrary standard. An arbitrary standard must have absorption in the visual spectrum sufficiently close to that of the pigment; in this case, we say that the pigment and the standard have the same color. When definite amounts of two pigments of the same color, each soluble in one or both phases, are introduced into the system, the intensity of the color of each phase will be fixed. Under certain conditions, a determination of the intensity of the color of both phases will permit an estimation of the total quantity of each pigment. These conditions are: (1) The distribution ratios of the pigments between the phases must differ considerably. We define the distribution ratio as the ratio of the concentration of the pigment in the upper (hexane) phase to that in the lower (alcohol) phase. In the system proposed, the distribution ratio for carotene, r_c, is about 60; that of xanthophyll, r_x, is about 0.03. (2) The distribution ratio of neither pigment should become reversed with change in concentration. This may happen in the case of an associating pigment. These two conditions must be established by direct observation of the behavior of each pigment separately. It should be emphasized that the method may be applied in certain cases where the distribution ratios are not constant, and where one pigment affects the distribution of the other. The obvious experimental procedure in all cases is to obtain the colorimetric readings of the phases for mixtures in which one pigment is varied at a time. From the results obtained, a graph may then be constructed from which, in suitable cases, the readings of the two phases will give directly the total amount of each pigment present. In the proposed system, we have found that a simple linear relationship holds between the colorimetric readings and the quantities of pigment present.

In devising an analytical method for pure pigments based on

these considerations, the following must be demonstrated: (1) that it is possible to estimate accurately the concentration of the pure pigments in the solvent components of the system by a colorimetric method; (2) that it is possible to form a system of two liquid phases between which the pure pigments become very differently distributed; (3) when a sufficiently large and varied series of mixtures of known amounts of the pure pigments is introduced into the system, that it is possible to construct a graph or to form an algebraic expression which will give *with accuracy* the quantity of each pigment corresponding to the appropriate pair of color values of the phases.

Materials

The carotene was obtained from the S. M. A. Corporation. It consisted almost wholly of β-carotene. We have had no experience with α- or γ-carotene, or with pure β-carotene. The carotene was recrystallized from hexane immediately before it was used. The crystals alter rapidly, as do the solutions. The alteration is evident from the fact that pigment passes more readily from the hexane phase into the alcohol phase of the systems studied. In fact, the distribution ratio may be used as a delicate test for the purity of carotene.

Xanthophyll was prepared from spinach leaves. We later obtained a supply from Dr. F. M. Schertz. The distribution coefficients did not differ. Before use, the xanthophyll was twice recrystallized from diacetone alcohol to which a trace of water had been added.

In preparing solutions for the quantitative studies of the carotenoids, the crystallizations were carried out in Pyrex centrifuge tubes. The drying took place at room temperature in vacuum desiccators containing sulfuric acid and blocks of paraffin. The concentrations as estimated by dissolving known weights of the pigments in known volumes of solvent agreed with those determined by the spectrophotometer by the method of Schertz.[1]

The hexane, Eastman Kodak Company, Practical, boiled between 62–67°. It was not purified further.

The petroleum ether, Baker's c.p., boiled below 60°.

[1] Schertz, F. M., *J. Agric. Research*, **26**, 383 (1924); **30**, 253 (1925).

The methyl alcohol was of c.p. grade.

The ethyl alcohol was distilled from alkali through a fractionating column.

The diacetone alcohol first used was a commercial brand, which formed a cloudy solution in water. We have found that the c.p. brand of the Eastman Kodak Company is sufficiently pure for use in the method. In time, a yellow color may develop. We have found it possible to purify diacetone alcohol by distillation at a pressure of 18 mm. of mercury, taking the fraction which boils between 70–71.5°. The refractive index of aqueous solutions of diacetone alcohol, as determined by the Zeiss dipping refractometer, was found to be a linear function of the concentration; if p is the concentration in volumes per cent, and n is the reading of the scale of the instrument at 20°, $p = 0.335n - 4.76$. In the method we use a solution prepared by adding 14 ml. of water to 100 ml. of diacetone alcohol. We check the concentration of this solution by diluting 2 ml. with water to 10 ml.; the scale reading of this mixture is 67.85; this gives as the concentration of the original solution 89.7 volumes per cent. A reduction in volume of 5.4 ml. occurs when 14 ml. of water are added to 100 ml. of diacetone alcohol.

Potassium Dichromate—Aqueous solutions of c.p. $K_2Cr_2O_7$ were used as colorimetric standards. The stronger stock solution contains 0.2 gm. per 100 ml.; the more dilute solution contains 0.02 gm. per 100 ml. Both solutions keep well.

Colorimetric Estimation of Carotene and Xanthophyll in Pure Solvents

Carotene was twice recrystallized, dried to constant weight, and dissolved in hexane. It was found impossible to dissolve a weighable quantity of carotene directly in a small volume of the various alcohols. Consequently, it was necessary to add accurately measured volumes of the solution in hexane to the alcohols. Since the color values of the resulting solutions differ only slightly from those in hexane, no appreciable error was introduced by the small quantity of hexane present. Solutions of xanthophyll twice recrystallized were prepared directly in the various alcohols. Small volumes of such solutions were diluted to known volumes with hexane when solutions in the latter solvent were needed. The

microcolorimeter was used; the solutions of carotenoid were set at depths of 5, 10, 20, and 30 mm. Precautions were taken to prevent evaporation of solvent. Unfiltered daylight was found more satisfactory than artificial light, with or without filters.

When the dilute standard (0.02 per cent $K_2Cr_2O_7$) was used, and when the concentration of carotenoid was below 1000 micro-
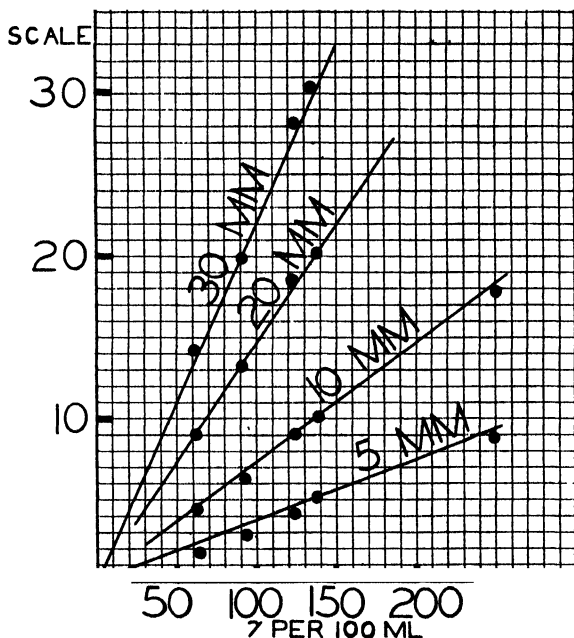
Fig. 1. Colorimetric estimation of carotene. The ordinates are readings of the dilute standard, 0.02 per cent $K_2Cr_2O_7$, in mm.; the abscissas are concentration of carotene, in micrograms per 100 ml. The several lines referring to settings of the unknown, in mm., are drawn so as to give the best fit to the entire set of data.

grams per 100 ml., the concentration was found to be directly proportional to the reading (R) of the standard, and inversely proportional to the depth (S) at which the solution was set: concentration, micrograms per 100 ml. $= K \times (R/S)$.

When the stronger standard was used, and when the concentration of carotenoid was above 1000 micrograms per 100 ml., the

linear relationship did not always hold.   In Fig. 1 is shown the set of readings for carotene in hexane when the dilute standard was used, and the lines for various settings, drawn with the value of K = 135.8, so determined as to give the least mean square deviation.

TABLE I

Color Constants, K, for Carotene and Xanthophyll for Use with Standards of Potassium Dichromate

Concentration in micrograms per 100 ml. $= K \dfrac{\text{reading of standard}}{\text{setting of solution}}$.

Dilute Standard, 0.02 Per Cent $K_2Cr_2O_7$

Solvent	Carotene K	Xanthophyll K
Hexane..	135.8	146.4
Diacetone alcohol.............................	135	145.2
Methyl alcohol................................	137	137

Concentrated Standard, 0.2 Per Cent $K_2Cr_2O_7$

Solvent	Carotene K	Xanthophyll K	Upper limits applicable		
			Setting of carotenoid	Reading of standard for	
				Carotene	Xanthophyll
			mm.	mm.	mm.
Hexane	1360	1500	30	5	4
			20	4	4
			10	4	3
			5	4	2.5
Diacetone alcohol	1350	1480	30	11	10
			20	8	10
			10	7.5	6.5
			5	3.5	3.5
Methyl alcohol	1360	1440	30	9	6
			20	5	5
			10	5	5
			5	3	3.5

In Table I are given the values of K for several solvents.   Values of K are also given for the stronger standard with the upper limits to which the linear relationship holds.   It is therefore evident that carotene and xanthophyll can be estimated by the colorim-

eter. In biological work, the most convenient setting of the colorimeter was found to be 30 mm. In the following parts of this paper we have therefore reduced the readings of the standard by the proper calculation to those which would have been found had the setting always been 30 mm. Such a calculation rests upon the facts established above. We speak of these readings as the "reduced readings." They may also be considered as the "color value" of the solutions.

Distribution of Carotene and Xanthophyll between Immiscible Solvents

Into tubes of about 10 ml. capacity, with narrow necks provided with cork stoppers, were measured 3 ml. of a solution of the carotenoid in hexane (or in alcohol) and 3 ml. of an alcohol (or hexane). The tubes were stoppered, placed in a water bath at 20°, and shaken at intervals for 10 minutes. After the layers had separated and become perfectly clear, the concentration of the carotenoid was determined, first in the upper layer, then in the lower. The ratios of these concentrations are recorded in Table II. It will be seen that these ratios are nearly constant.

Selection of Components of System Suitable for Method—As already stated, the purpose of the preliminary observations was to select a system in which most of the carotene would be in the hexane phase, while most of the xanthophyll would be in the alcohol phase—that is, a system for which r_c is large and r_x small. The data in Table II show that r_c and r_x are not greatly affected by the total amount of pigment. In the further study of suitable conditions it was therefore not necessary to observe more than a single example of each system. It will be seen in Table II that both r_c and r_x may increase when water is introduced into the system. The effect of water is more fully illustrated in Table III. The results were obtained by distributing carotene and xanthophyll in 2 ml. of hexane, 2 ml. of acetone or alcohol, and amounts of water varying from 0.05 to 0.7 ml. at 20°. These observations indicate that acetone is not a suitable component because r_x is large. Methyl and ethyl alcohol are inferior to diacetone alcohol because r_x is smallest in systems containing the latter. We have therefore selected diacetone alcohol for use in the method. The selection of any particular quantity of water is somewhat

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TABLE II

Distribution Ratios of Carotene and Xanthophyll at 20°; Upper Phase,
Hexane

</div>

Concentration per 100 ml. phase		Ratio of concentrations	Components of lower phase
Hexane	Alcohol		
Carotene			
micrograms	*micrograms*		
10,720	85.2	126	Ethyl alcohol, 75% by weight
743	5.83	128	
289	13.8	20.9	Diacetone alcohol (impure), 100
144	5.82	24.7	ml., + water, 10 ml.
73.7	3.04	24.2	
1,165	10.0	117	Diacetone alcohol (impure), 100
454	4.0	113	ml., + water, 14 ml.
Xanthophyll			
171	512	0.33	Ethyl alcohol, 75% by weight
71.5	206	0.35	
51	137	0.37	
31.5	82.5	0.38	
21	54.5	0.39	
91.5	376	0.24	Methyl alcohol, 100 ml., + water,
46.5	184	0.25	13.75 ml.
23.1	93	0.25	
11.6	47.5	0.24	
6.0	24.0	0.25	
3.0	12.5	0.24	
8.09	334	0.024	Diacetone alcohol (impure), 100
3.81	161.5	0.024	ml., + water, 10 ml.
1.56	82.2	0.019	
0.88	41.3	0.021	
20.7	311	0.067	Diacetone alcohol (impure), 100
9.78	153	0.064	ml., + water, 14 ml.
4.89	75.9	0.065	
2.45	37.7	0.065	
12.2	376	0.033	Diacetone alcohol (pure), 100 ml.,
4.0	115	0.035	+ water, 14 ml.
1.3	36	0.035	

arbitrary. In practice we have used a system comprised of 2 ml. of hexane and 2 ml. of an aqueous diacetone alcohol made by mixing 14 ml. of water and 100 ml. of the alcohol. For this system,

TABLE III

Effect upon Distribution Ratios of Carotene and Xanthophyll of Adding Water to System Composed of 2 Ml. of Hexane and 2 Ml. of Acetone or an Alcohol at 20°

Water, ml.		0.05	0.10	0.15	0.20	0.25	0.3	0.4	0.7
Acetone	r_c	Separate layers do not form			14.2			∞	∞
	r_x				5.7			9	20
Methyl alcohol	r_c	27.8	82.0	124	1090	1590	15,500	∞	∞
	r_x	0.26	0.29	0.28	0.40	0.35	0.39	0.53	
Ethyl alcohol	r_c	5.5	10.5	27.3	68.1	91.4	183	2510	∞
	r_x	0.33	0.22	0.21	0.22	0.23	0.24	0.27	0.36
Diacetone alcohol	r_c	3.45	9.5	26.4	48	71.8	119	357	800
	r_x	0.069	0.043	0.040	0.035	0.049	0.063	0.095	0.36

r_c represents the ratio of the concentration of carotene in the upper phase to that in the lower; r_x represents this ratio in case of xanthophyll.

the volumes of the resulting phases are very nearly 2 ml. at 20°
in each case.

Selection of Volumes of Components—In studies of the caroten-
oids, it is usually convenient to bring the mixed pigments into
solution in light petroleum ether.   When the material being ana-
lyzed is limited in amount, a volume of 2 ml. is convenient for
observation in the microcolorimeter.   If the solvent is removed by
evaporating 2 ml. of such a solution and if the pigments are then
distributed between the phases of the proposed system, we obtain
approximately 97 per cent of the carotene in the hexane phase,
and 97 per cent of the xanthophyll in the alcohol phase.

Errors in observation of the color values of the phases obviously
result in errors in the calculated values of carotene and xantho-
phyll.   It can be shown that if r_c is large and r_x is small, the ratio
of volumes of the phases has little effect upon the magnitude of
this error.   In case r_c and r_x are more nearly alike, the magnitude
of the error in question is appreciably affected by the ratio of the
volumes of the phases.   In this case, it can be shown that the
error will be least when the ratio of the volume of the alcohol phase
to that of the hexane phase is made equal to $\sqrt{r_c r_x}$.   This fact
deserves mention because the method may find application to cases
where the two distribution coefficients do not differ as much as in
the case of carotene and xanthophyll.

Analysis of Mixtures of Carotene and Xanthophyll

Solutions of carotene and of xanthophyll were made in hexane.
The exact concentration was determined with the colorimeter.
Mixtures were prepared with varying composition, as shown in
Table IV under "Carotene present," and "Xanthophyll present."
2 ml. of each solution or mixture were thoroughly agitated with
2 ml. of aqueous diacetone alcohol at 20° in a closed tube, and the
color value of each of the resulting phases was determined with the
colorimeter.

The algebraic expression of these data is based upon the fol-
lowing facts established earlier in this paper with regard to the
pure pigments: (1) The concentration of pure carotene or pure
xanthophyll in either phase is directly proportional to the color
value of the phase.   (2) The distribution ratios for each pigment
are approximately constant; that of carotene is large, that of

xanthophyll is small. Therefore, when carotene alone is present, its *original concentration* must be directly proportional to the color value of either phase; and when xanthophyll alone is present,

TABLE IV

Analysis of Mixtures of Carotene and Xanthophyll

The calculations were carried out with the equations $C = 4.57U - 0.303L$ and $X = 5.14L - 0.0366U$ (where C and X represent carotene and xanthophyll, and U and L the upper and lower phases). The numerical coefficients were calculated by the method of least squares to give the best fit of the equations to the data for pure carotene (Solutions a_1, a_2, a_3, and a_4) and for pure xanthophyll (Solutions e_1, e_2, e_3, and e_4).

Results for carotene and xanthophyll are expressed in micrograms per 100 ml.

Solution	Reduced readings		Carotene		Xanthophyll	
	Upper phase	Lower phase	Present	Calculated	Present	Calculated
	mm.	*mm.*				
a_1	60.72	0.46	278	277.4	0	+0.2
a_2	30.57	0.23	139	139.6	0	+0.1
a_3	15.68	0.1	70.5	71.6	0	−0.1
a_4	7.78		34.9	35.5	0	0
b_1	55.38	5.42	251.2	251.4	25.7	25.9
b_2	27.69	2.74	126.9	126.0	13.0	13.0
b_3	13.94	1.28	64.1	63.4	6.55	6.1
b_4	6.96	0.72	31.9	31.6	3.3	3.4
c_1	31.59	27.33	134.0	136.2	141.0	139.7
c_2	16.12	13.50	67.3	69.7	71.3	69.0
c_3	7.86	6.66	33.3	34.0	35.3	34.0
c_4	3.81	3.38	16.1	16.4	17.2	17.2
d_1	9.52	49.78	24.5	28.4	258	255.7
d_2	4.66	24.72	12.3	13.8	129	127.5
d_3	2.34	12.60	6.1	6.9	64.0	65.0
d_4	1.22	6.28	3.0	3.7	31.6	33.0
e_1	4.16	62.88	0	+0.1	324	323.8
e_2	2.00	31.46	0	−0.3	161.2	161.9
e_3	1.00	15.64	0	−0.1	81.5	80.6
e_4	0.50	7.76	0	0	40.8	40.0

its *original concentration* must be directly proportional to the color value of either phase. If we further assume that neither pigment affects the distribution of the other and that the color values are additive, it can be shown that the original concentration of each

pigment is given by the simple linear equations: $C = aU - bL$; $X = cL - dU$, where C and X are the original concentrations of carotene and of xanthophyll in micrograms per 100 ml., U and L are the color values (reduced readings in mm.) of the upper and of the lower phases, and a, b, c, and d are constants which hold for the entire set of data. Moreover, since most of the carotene is in the upper phase, and most of the xanthophyll is in the lower phase, it is evident that the second term in each equation must

TABLE V

Data for Determination of Constants in Equations for Estimation of Carotene and Xanthophyll

$C = 4.62U - 0.125L$ and $X = 5.03L - 0.0827U$.

The results for the color values are expressed in mm.; for carotene and xanthophyll, in micrograms per 100 ml.

Solution	Color values		Carotene		Xanthophyll	
	Upper phase	Lower phase	Present	Calculated	Present	Calculated
a_1	85.50	1.38	394.8	394.8	0	−0.1
a_2	42.54	0.76	198.0	196.7	0	−0.3
a_3	21.33	0.32	98.4	98.5	0	+0.2
a_4	10.66	0.16	49.4	49.3	0	+0.1
a_5	5.40	0.08	24.8	24.9	0	0
e_1	2.48	91.74	0	0	459.5	460.8
e_2	1.30	45.36	0	+0.2	230.2	227.9
e_3	0.64	22.77	0	−0.1	114.6	114.4
e_4	0.32	11.56	0	0	58.4	58.4
e_5	0.16	5.68	0	0	28.8	28.6

have a small coefficient and may therefore be regarded as a correction term.

The coefficients a, b, c, and d were determined from the data for the pure pigments (Solutions a_1, a_2, a_3, a_4, and c_1, c_2, c_3, and c_4) so as to give the least mean square deviation of the differences between the concentrations actually present and those calculated. With the coefficients so determined, the values of C and X were calculated. The agreement is as good as might be expected in a colorimetric method. By including the data for the mixtures as well as those for the pure pigments, slightly different coefficients were obtained; these coefficients gave somewhat smaller differ-

ences than those recorded in Table IV between the values of C and of X, present and calculated. This better fit is of course a mathematical consequence of the theory of errors. We have a strong feeling that the larger errors in the case of the mixtures are no more likely to be due to erroneous theory than to difficulty in making the mixtures accurately.

We may therefore conclude that for the pigments carotene and xanthophyll in the system proposed, the simple linear equations hold as stated above, and that the constants of these equations may be derived from the data obtained with pure pigments. This
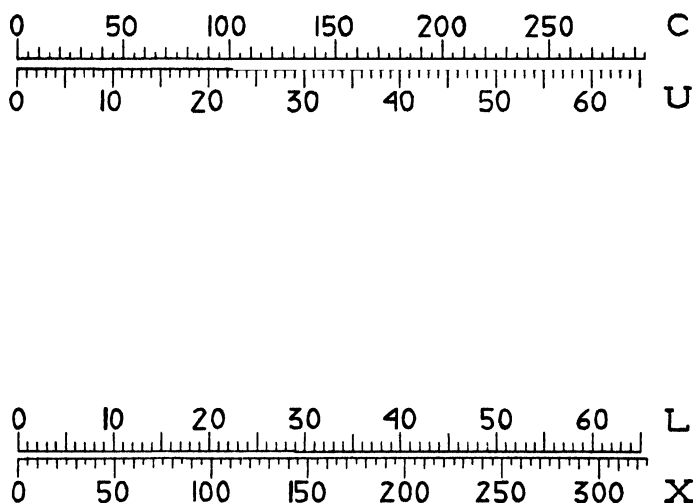
FIG. 2. Nomogram for estimation of carotene and xanthophyll. U and L are the reduced readings, in mm., of the upper (hexane) phase and lower (diacetone alcohol) phase when 0.02 per cent $K_2Cr_2O_7$ is used as standard. A straight line joining the reduced readings meets the scales C and X at points indicating the concentration of carotene and of xanthophyll, in micrograms per 100 ml.

procedure obviously eliminates the experimental errors introduced in making mixtures, and goes far towards eliminating errors due to evaporation of solvent.

The experiment described above was carried out with a commercial sample of diacetone alcohol. For the determination of the constants for use in the method we have used highly purified di-

acetone alcohol (Table V). We have applied these constants with satisfactory results in the estimation of known mixtures of the pigments. For ease in carrying out the calculations, a nomogram (Fig. 2) is used. A straight line joining the color values of the upper and of the lower phase, U and L, indicates on scales C and X the original concentration of carotene and of xanthophyll.

Application of Method to Analysis of Biological Material

We have demonstrated above that the proposed method applies to the analysis of mixtures of pure carotene and xanthophyll. When the method is to be applied to the estimation of carotene and xanthophyll in mixtures which contain other substances, it is necessary to prove that no other pigments are present, that the colorless substances present do not affect the estimation, and that the various procedures carried out in the course of the analysis do not alter the pigments. We have not yet applied the method to the analysis of extracts of leaves, fruits, vegetables, or cereals. The method was developed for the study of the carotene and xanthophyll content of blood, liver, and other animal tissues. In this paper we can give only briefly the results of our observations on the application of the method to the analysis of blood plasma and serum.

Preparation of Extracts of Plasma or Serum—1 volume of plasma or serum is mixed with 1 volume of 95 per cent ethyl alcohol and 1 volume of light petroleum ether in a tightly stoppered tube. The mixture is shaken vigorously at intervals for 10 minutes. The supernatant layer is separated with the centrifuge. Carotenoid pigments are completely extracted from the lower layer. This is shown by the fact that a second extraction removes no more pigment; regarding distribution ratios the data in Table II indicate that this result is to be expected. Moreover, the volume of the supernatant fluid is found by experiment to be equal to that of the original plasma or serum. Consequently, the carotenoids are present in it in the same concentration as in the original plasma. Great care must be exercised to prevent evaporation of the solvent.

Nature of Pigments in the Extract—Bile pigment is not present in the extract. So far as we have been able to ascertain, all the color of the extract is probably due to carotenoid pigments.

These pigments have been separated from the original extract by suitable solvents. We have obtained a fraction which has the distribution ratio between hexane and diacetone alcohol, and the spectrophotometric absorption curve characteristic of xanthophyll. We do not know whether pigmented oxidation products of carotene are present in the extract; this is not unlikely. Esters of xanthophyll are present in small amounts only. A fraction which has the distribution ratio characteristic of carotene is also present; but the spectrophotometric absorption curve indicates that several substances are often present, chiefly β-carotene, sometimes lycopene.

In summary, we may state that by the proposed method of analysis, we include under the term "carotene," true carotene, lycopene, and xanthophyll esters; and under the term "xanthophyll," free xanthophyll and possibly oxidation products of carotene.

Effect of Colorless Substances in the Extract—The extract may contain phospholipid, cholesterol and its esters, and fat. Lecithin, cholesterol, and fat (olive oil) in concentrations as high as 10 times those likely to be present in plasma were found to have no effect on the distribution ratios of carotene and xanthophyll. We believe that this indicates that the colorless substances likely to be present in the extract do not affect the estimation.

Alteration of Carotene and Xanthophyll during Analytical Procedures—We find little, if any, loss of carotenoids in samples of plasma or serum kept in a dark refrigerator for a week. The petroleum ether extracts kept at room temperature fade somewhat within several hours. Saponification invariably causes considerable loss. Evaporation at room temperature in a current of dry air, or nitrogen, leads to little, if any, alteration. Solutions of the *pure* pigments are altered by evaporation. This change can be largely prevented by a trace of hydroquinone. In view of these observations, it is obvious that the petroleum ether may be removed from the extract of plasma, and probably of other tissues, by rapid evaporation at room temperature; and that the analysis should be completed within a few hours.

Directions for Carrying Out Method—The mixture to be analyzed should be dissolved in hexane. If petroleum ether extracts are available, a measured volume is freed of solvent as directed above,

and the residue dissolved in the same volume of hexane.   To the solution, contained in a tube with a narrow neck, fitted with a tight cork stopper, is added an equal volume of aqueous diacetone alcohol.   The latter solution is prepared by mixing 100 ml. of pure diacetone alcohol with 14 ml. of water.   The tube is placed in a beaker of water at 20° and is shaken at intervals for 10 minutes. After the layers have separated and have become perfectly clear, a portion of the upper layer is transferred to the cup of the micro-colorimeter, the plunger of which is set at 5, 10, 20, or 30 mm.   A series of five concordant readings is taken with 0.02 per cent potassium dichromate as standard.   The mean is multiplied by 6, 3, or 1.5 to obtain the color value of the upper phase (the "reduced" reading).   The excess of upper phase is removed from the tube by means of filter paper and the color value of a portion of the lower phase is determined in the colorimeter.

From the color values so obtained, the original concentrations of carotene and xanthophyll in the petroleum ether or hexane solution in micrograms per 100 ml. are calculated by means of the equations $C = 4.62U - 0.125L$; $X = 5.03L - 0.083U$, or with the nomogram (Fig. 2).   In the latter case, a line joining the color value of the upper phase, on scale U, to the color value of the lower phase, on scale L, meets scale C and scale X in points indicating the concentrations of carotene and of xanthophyll.

SUMMARY

1. A general theory is outlined for the quantitative estimation of two substances having the same color by a single distribution between two immiscible liquids.

2. The conditions are established for the application of the theory to the analysis of mixtures of pure carotene and xanthophyll.

3. The necessary precautions are given which must be taken when other substances are present.

4. The method is applied to the analysis of blood plasma and serum.

THE PROVITAMIN D OF HEAT-TREATED CHOLESTEROL*

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As early as 1928 the work of Bills, Honeywell, and MacNair (1) demonstrated that cholesterol purified by various methods and apparently free from ergosterol still possessed provitamin D activity. This was confirmed by Koch, Koch, and Ragins (2) and it was also shown (2, 3) that cholesterol purified through the dibromide and then heated at 200° for 2 hours with traces of oxygen showed greatly increased activatability.

Waddell's report (4) on the provitamin D of cholesterol for the chick and Bills' evidence (5) that the vitamin D content of fish oils is different when assayed for rats and for chicks have stimulated interest in the multiplicity of forms of vitamin D. The next step was to test on chicks the provitamin D produced by heat treatment of the cholesterol. It is the purpose of this paper to report the results of preliminary experiments on this point.

EXPERIMENTAL

The basal ration, methods of feeding, and methods of ashing recommended by Hart, Kline, and Keenan (6) were used except that all the chicks received the unsupplemented basal diet for the 1st week followed by the supplemented diet for a 4 week period. Day-old white Leghorn chicks from Storrs Agricultural College at Storrs, Connecticut, were used in these studies.

The supplements used were reference cod liver oil (95 U.S.P. units per gm.), irradiated ergosterol in maize oil (Mead Johnson

and Company), crude cholesterol (Wilson and Company), purified cholesterol, and heated purified cholesterol. The last two samples were prepared as follows: crude cholesterol was purified through the dibromide (1); samples of this purified product were heated in an oil bath at 200° for 2 hours in a small flask with two capillary openings and then recrystallized from hot alcohol. All the cholesterol samples were irradiated in thin layers under a Cooper Hewett mercury arc lamp for 35 minutes at 40 cm.

A preliminary test was run in which the chicks, in groups of four, were fed directly into the crop 1 Steenbock rat unit (or 2.7

TABLE I

Preliminary Assay of Cholesterol Samples for Provitamin D Activity

Group No.	Supplement to basal ration	Steenbock rat units per day*	Weight at 35 days	Gain in weight during supplementary feeding	Bone ash	Condition of chicks
			gm.	*gm.*	*per cent*	
I	None (controls)	0	123	74	30.3	All squat at 3 wks.
II	Irradiated crude cholesterol	1	181	127	44.6	All very active
III	Irradiated purified cholesterol	1	147	85	33.2	" squat at 3 wks.
IV	Irradiated heated, purified cholesterol	1	176	118	44.7	All very active

* 1 Steenbock unit = 2.7 international or u.s.p. units.

u.s.p. units) per day of vitamin D supplement in 0.6 cc. of maize oil for a 4 week period. The basal diet and water were supplied *ad libitum.* Three supplements were used in this preliminary study: (1) irradiated crude cholesterol, (2) irradiated purified cholesterol, and (3) irradiated heated, purified cholesterol. By rat assay it was shown that for the irradiated crude cholesterol and the irradiated heated, purified product 0.5 mg. was equivalent to 1 Steenbock rat unit, and for the irradiated purified cholesterol 10.0 mg. were required for the 2+ cure. The results on the chicks are given in Table I.

In the second set of experiments the vitamin D was added to

TABLE II

Comparative Potency of Vitamin D from Different Sources for Chicks

Group No.	Supplement to basal ration	Supplement per 100 gm. of ration		Average weight at 35 days	Average gain in weight during supplementary feeding	Bone ash	Condition of chicks
		Steenbock rat units	U.S.P. units				
				gm.	*gm.*	*per cent*	
I	None (controls)	0	0	138	69	31.1	Unsteady in 2½ wks.; all squat at end of 3rd wk.
II	0.147 gm. % cod liver oil	5	13.5	259	191	42.5	Excellent, very active
III	0.294 gm. % cod liver oil	10	27.0	271	202	44.1	" "
IV	10.8μ gm. % irradiated ergosterol*	100	270.0	200	130	40.6	Fair, unsteady last wk.
V	32.4μ gm. % irradiated ergosterol*	300	810.0	247 (135)	179 (79)	47.1 (44.6)	Good, very active (2 chicks ill last wk.)†
VI	0.0025 gm. % irradiated crude cholesterol	5	13.5	241	173	46.2	Excellent, very active
VII	0.005 gm. % irradiated crude cholesterol	10	27.0	253	183	45.9	" "
VIII	0.2 gm. % irradiated purified cholesterol	20	54.0	187	116	36.5	Poor, all unsteady last wk.
IX	0.0025 gm. % irradiated heated, purified cholesterol	5	13.5	222	149	41.7	Excellent, very active
X	0.005 gm. % irradiated heated, purified cholesterol	10	27.0	273	199	45.8	" "

* Group IV supplement contained 0.27 mg. per 100 gm., and Group V supplement 0.81 mg. per 100 gm. of a solution of irradiated ergosterol in maize oil, containing 1,000,000 international units of vitamin D per gm. and about 0.04 gm. of irradiated product per cc., furnished us by Mead Johnson and Company.

† In Group V the second reading applies in each instance to the two chicks that were ill the last week.

maize oil so that 1 gm. of oil per 100 gm. of food gave the desired potency. Table II gives the supplements used and the number of units of each which were fed. At the end of 5 weeks the chicks were killed; the tibiæ were removed and extracted and ashed by groups. The average results for the five chicks per group are given in Table II.

<div align="center">DISCUSSION</div>

It was evident from the preliminary assay (Table I) that 1 Steenbock rat unit of irradiated crude cholesterol per day entirely protected the chick from rickets, but that the equivalent in rat units of irradiated purified cholesterol was ineffective. When the purified product was treated under definite conditions, a fraction was formed which on irradiation was again effective for the chick.

In Table II further assays are reported to show the relative antirachitic value of various sources of vitamin D. It is evident that chicks on cod liver oil, crude cholesterol, or heated purified cholesterol at a level of 10 Steenbock rat units (27 international or U.S.P. units) per 100 gm. of food were adequately protected, and that at a level of 5 rat units the dose was not sufficient except in the case of crude cholesterol, although all the chicks on the supplements were improved (showing bone ash of 41.7 per cent). The results from administration of 100 rat units of irradiated ergosterol were about equivalent to 5 rat units of heated purified cholesterol, and those fed 300 rat units per 100 gm. of ration corresponded to the 10 rat unit group.

The work of Bethke, Record, and Kennard (7), of Murphy, Hunter, and Knandel (8), and of Carver, Robertson, Brazie, Johnson, and St. John (9) shows that 17 international units of vitamin D from cod liver oil per 100 gm. of our diet are required for the growing chick. Our work roughly confirms this for the vitamin D of cod liver oil and for irradiated heated cholesterol, since we found 27 units to be adequate, but 13.5 units were somewhat below the requirement. We also confirmed the evidence (7, 10–14) that at least 20 times this amount of vitamin D from ergosterol is required for an equivalent cure.

The results have confirmed the work of Waddell (4) that irradiated crude cholesterol is many times more effective on chicks,

rat unit for rat unit, than irradiated ergosterol.   It should be noted that for the particular sample we used the lowest level fed, 5 rat units (or 13.5 u.s.p. units) per 100 gm. of ration, was entirely adequate, showing it as more effective, rat unit for rat unit, than the particular cod liver oil sample we used in our assay.

Our results also show that the provitamin D, still present in cholesterol purified by way of the dibromide, is less effective, rat unit for rat unit, than that of the crude cholesterol, but that the same or a different provitamin D, effective for chicks, is found on heating this purified cholesterol under definite conditions.   To explain the apparent discrepancy between our results and those of Dr. Waddell on heat-treated cholesterol this difference should be pointed out.   He treated only samples containing cholesterol to which 5 per cent ergosterol had been added, and since the added ergosterol reduced the rat unit to 0.011 mg. and 0.02 mg. of the irradiated mixture, the amounts fed did not contain enough of the heat-treated cholesterol to show the increase in provitamin due to the heating.   Our subminimal dose was 2.5 mg. and the adequate dose 5 mg. per 100 gm. of food.

The work of Windaus and Lüttringhaus (15) on the structure of ergosterol shows that ergosterol contains one more CH_2 group in the side chain than is found in cholesterol.   It is certainly inconceivable that simply heating cholesterol slightly above the melting point could bring about such a change in molecular structure.   Therefore on the basis of our study we conclude that a new form of vitamin D has been formed by the heat treatment of cholesterol, which corresponds, rat unit for rat unit, to the vitamin D of cod liver oil for chicks.   Further work on concentration and identification of the provitamin is in progress.

SUMMARY

1. Waddell's observation that irradiated crude cholesterol is more effective in preventing rickets in chicks than an equivalent number of units of irradiated ergosterol has been confirmed.

2. This provitamin of crude cholesterol is destroyed by purification through the dibromide; *i.e.*, 20 rat units or 200 mg. of purified cholesterol per 100 gm. of ration showed only slight improvement over the basal diet alone.

3. Heat treatment of the purified cholesterol increases its proantirachitic potency so that 2.5 to 5 mg. per 100 gm. of ration completely protect the chick.

4. Evidence is given that irradiation of heat-treated cholesterol has formed a new form of vitamin D which has properties resembling those of the natural vitamin D of cod liver oil more closely than those of the vitamin D of irradiated ergosterol, as shown by chick assay.

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STUDIES IN SERUM ELECTROLYTES

X. THE WATER OF SERUM AND FACTORS FOR THE CALCULATION OF THE MOLALITY OF A SOLUTE IN SERUM FROM THE MEASUREMENT OF THE SPECIFIC GRAVITY

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Recent studies have demonstrated the desirability in certain instances of expressing the concentration of a solute in serum in relation to the concentration of water. For this relationship the measurements of both the specific gravity and the amount of total solids have been necessary. In this paper it will be shown that since there is a linear correlation between the specific gravity and the total solids, the measurement of either of these will suffice for the calculation of the molality of a solute in serum with sufficient accuracy for most purposes. In addition, the amount of water present in serum may be calculated directly from the measurement of the specific gravity alone.

The specific gravity of 183 specimens of serum was plotted against the gm. of water per kilo of the respective sera. The specific gravity measurements were made at 20° with pycnometers of 2 ml. capacity. The amount of total solids was determined by drying a weighed amount of serum at 100–105° to constant weight. The water per kilo of serum was calculated from the measurement of the total solids. All of the measurements were made on human sera obtained from patients suffering from miscellaneous pathological conditions. The sera were removed without hemolysis from centrifuged specimens of the clotted blood.

The statistically calculated regression line of water on specific

gravity, shown as a solid line in Fig. 1, may be expressed by Equation 1.[1]

(1) $H_2O/K = 4082.3 - 3086.3\ Sp$

The standard deviation from this regression line is equal to 3.5. Theoretically the limiting value of the line should extrapolate to a specific gravity of 1.000 when the H_2O is 1000.   The dotted line
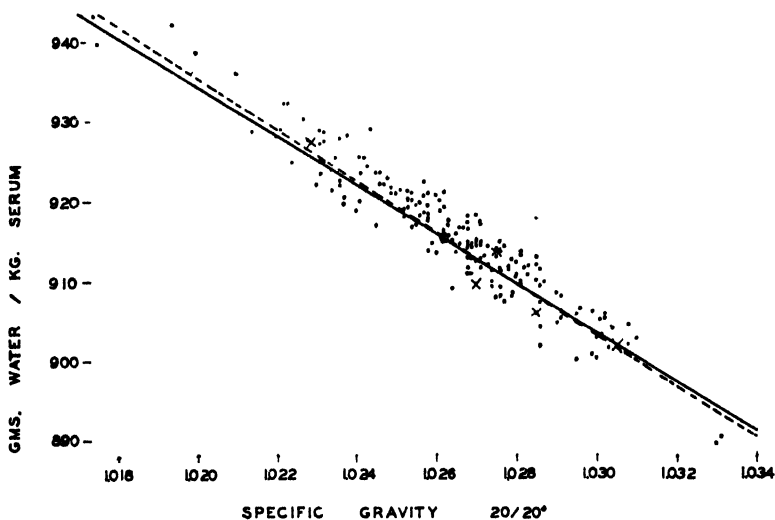
FIG. 1. The water of serum plotted against the specific gravity

shown on Fig. 1 is corrected to pass through this limiting value and may be expressed by Equation 2.

(2) $H_2O/K = 4225.6 - 3225.6\ Sp$

Since the corrected regression line is well within twice the scattering of the data and since the water values obtained by Equa-

[1] In the calculations the following relationships are used

$$X/K = (X/L)/Sp;\ H_2O/K = 1000 - S/K;\ [X] = \frac{X/L \cdot 1000}{H_2O/K \cdot Sp}$$

where Sp represents specific gravity at 20°, H_2O water in gm., S solids in gm., X a solute in serum, L liters of serum, K kilos of serum, [ ] concentration per kilo of water.

tion 2 over the range of the specific gravity values observed in serum are within ±0.2 per cent of those obtained by Equation 1, Equation 2 is employed instead of Equation 1.

In Table I are given the factors by which for any serum specific gravity the concentration of a solute per liter of serum may be calculated as the concentration per kilo of water. In addition,

TABLE I

Serum Water and Factors for Calculation of Molality of a Solute (X) in Serum When Specific Gravity Has Been Measured

Sp. gr. 20°/20°	Water per kilo serum	Water per liter serum	Multiplying factors for conversion of X per liter serum to X per kilo H_2O in serum
	gm.	*gm.*	
1.015	952	966	1.035
1.016	948	964	1.038
1.017	945	961	1.040
1.018	942	959	1.043
1.019	939	957	1.045
1.020	936	954	1.048
1.021	932	952	1.051
1.022	929	949	1.053
1.023	926	947	1.056
1.024	923	945	1.059
1.025	919	942	1.061
1.026	916	940	1.064
1.027	913	938	1.067
1.028	910	935	1.069
1.029	906	933	1.072
1.030	903	930	1.075
1.031	900	928	1.078
1.032	897	925	1.081
1.033	894	923	1.083
1.034	890	921	1.086

the gm. of water and solids per kilo or per liter of serum may be obtained directly from Table I for any serum specific gravity.

It would seem apparent that the close parallelism between the specific gravity and the solid content of serum would not apply to lipemic serum displaying a frank lactescence with separation of the fats. · In one such specimen, excluded from Fig. 1, the total solids were 149.5 gm. per kilo of serum; the total fats, 8.8 gm. per

100 ml.; the cholesterol, 1298 mg. per 100 ml.; and the specific gravity, 1.018.   On the other hand, the correlation would appear to hold satisfactorily in sera containing at least moderate elevations in the cholesterol content.   In the sera marked $\times$ on Fig. 1, the cholesterol values ranged from 250 to 450 mg. per 100 ml.   No unusual deviation from the regression line was observed in these specimens.

<div style="text-align:center">DISCUSSION</div>

The molal concentration of a solute and the partial molal solute quantity are the relationships frequently used in thermodynamic calculations, although it should be pointed out that in measurements made on simple aqueous solutions and treated by the Debye-Hückel equation, the use of the volume concentration of the solute is essential.   In the case of complex biocolloidal solutions containing a large solid phase the concentration of a solute per unit of solvent appears to have especial significance in relation to the colligative properties of the solute.   Thus, for example, it has been shown (1) that the observed freezing point depression of serum to which a solute, such as sucrose or NaCl, has been added is in agreement with the calculated depression, provided the concentration of the added solute be calculated per unit of water in the serum. The studies of Van Slyke, Wu, and McLean (2) indicate that when the concentrations of the electrolytes, regardless of species, are expressed as moles of ions per kilo of water, the same concentration of ions is present in the cells of the blood as in the serum.   Moreover, further evidence may be cited in the equal distribution of sugar between the serum and the blood cells when the molal concentrations of this component are compared (3, 4).

The specific gravity of serum and plasma is used clinically in the estimation of the protein content with a maximum error, according to Moore and Van Slyke (5), of 0.6 gm. per 100 cc. of plasma.   Since the protein content of serum is between 75 and 80 per cent of the dry weight, a correlation between the specific gravity and dry weight might have been expected.

McLean and Hastings (6) calculate the amount of water per 100 cc. of serum from the measurement of the protein.   They assume that 1 per cent of the volume of serum represents solids other than protein and adopt Svedberg and Sjögren's (7) value of 0.75 as the

specific volume of the serum proteins. The calculated values of water by this method and by the specific gravity method are in close agreement.

SUMMARY

A linear correlation has been demonstrated between the specific gravity and the total solids of serum. After determination of either of these two measurements, a solute measured in relation to volume may be calculated in relation to the water with an accuracy of ± 1 per cent. In addition, the concentration of the water present in serum may be derived directly from the measurement of the specific gravity.

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DETERMINATION OF FREE AND COMBINED CHOLESTEROL IN BILE*

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During the course of studies on gallbladder function being carried on in this laboratory it became desirable to determine both free cholesterol and ester cholesterol, if any were present, in bile. The only available procedure for differentiating between free and ester cholesterol involves precipitation of free cholesterol as the digitonide, and it soon became apparent that none of the methods available was wholly applicable to bile. We, therefore, attempted in various ways to modify the procedures described for determining cholesterol ester in order to devise if possible a method applicable to bile which would offer both accuracy and ease of execution. The method finally adopted was a modification to an extent of the Schoenheimer-Sperry procedure for determining cholesterol in blood (1).

Reagents

Magnesium sulfate, 15 per cent. 100 gm. of $MgSO_4 \cdot 7H_2O$ dissolved in 220 cc. of water.

Alcohol-acetone 1:1. 1 volume of 95 per cent ethyl alcohol to 1 volume of C.P. acetone.

Phenol red, 0.02 per cent, in 50 per cent alcohol.

Hydrochloric acid, 5 per cent. 1 volume of concentrated HCl to 7 volumes of water.

Digitonin reagents. (a) 0.7 per cent in water. 3 to 5 cc. of water are added to 1 gm. of digitonin (Hoffmann-La Roche) in a beaker. This is stirred while heating on a water bath until clear. To this are then added with stirring 130 cc. of hot water. (b) 0.5 per cent alcoholic digitonin. This presents no difficulties.

Acetic acid, 100 per cent (Eastman).

Acetic anhydride (Eastman).

Sulfuric acid, concentrated c.p.

Potassium hydroxide, 33 per cent.

Acetone-ether. 1 volume of c.p. acetone to 2 volumes of c.p. ether.

Standard Solutions—The color developed by the cholesterol digitonide differs from that developed in a pure cholesterol standard of equivalent cholesterol content. It is essential, then, that the standard solutions used for comparison also contain digitonin.

Standard A—Known amounts of a standard chloroform solution of cholesterol are measured into test-tubes. Where the range of amount in the bile specimens is unknown, it is best to prepare several standards (*e.g.,* 1, 2, 3, 5 mg.). The chloroform is evaporated by heating on a water bath at approximately 65°. To the dry residue is added 0.7 cc. of 0.5 per cent alcoholic digitonin solution for each mg. of cholesterol. This is then evaporated to dryness on the water bath at 85°. A large number of standard tubes may be prepared in this way, and then kept for use indefinitely. They should be thoroughly dried again before use.

Standard B—A solution containing a known amount (we use 0.75 gm. of cholesterol digitonide per 100 cc.) of cholesterol digitonide in acetic acid may be prepared. 0.5, 1.0, 2.0, and 3.0 cc. of this are pipetted into test-tubes, and made up to a volume of 3.0 cc. with acetic acid.

By use of Standard B certain variations in color development are eliminated, and it is felt that the use of this standard increases the accuracy of the method.

Cholesterol Digitonide—Solution A, 1.2 gm. of cholesterol in 15 cc. of absolute alcohol; Solution B, 3.18 gm. of digitonin dissolved with warming in 50 cc. of absolute alcohol, small portions being added at a time. Add the warm Solution B to Solution A, washing Solution B into Solution A with 10 cc. of hot absolute alcohol. Place on the steam bath and if necessary filter hot through an ether-washed gauze plug. Wash twice with 10 cc. of hot absolute alcohol. Allow to cool slowly to 40°. Filter off the precipitate which forms. Add slowly 10 cc. of acetone and again filter. The filtrate is placed in two 50 cc. centrifuge tubes, and to each are added, drop by drop with stirring, 5 cc. of water. A heavy white precipitate forms. Place the tubes in a water

bath at 80° for 15 minutes. The tubes are then centrifuged, the supernatant fluid removed, and each tube washed successively with (a) 25 cc. of alcohol-acetone 1:1, (b) 25 cc. of acetone–ether, (c) twice with 40 cc. of ether, the precipitate being stirred up each time and the tube again centrifuged. The last washing should give only a faint test or a negative test for cholesterol. Dry the residue to constant weight at 100°.

Test solutions were prepared by dissolving 0.4 gm. of the dried residue in 100 per cent acetic acid, with warming, and made up to 50 cc. in a volumetric flask.

These solutions were tested against standards made up of cholesterol as in Standard A and also against known amounts of cholesterol run through the complete digitonide analysis. Theoretical cholesterol concentrations were calculated by multiplying the total digitonide concentration by the factor $386.4/1601 =$ 0.2414.

Method

A known amount of bile is measured into a round-shouldered liter bottle, and water added to a volume of 10 cc. 1 cc. of 15 per cent magnesium sulfate and 100 cc. of ether are added and the mixture shaken 20 minutes (we use a Kahn shaking apparatus with a platform on top containing brackets of the correct size to hold the bottles). After settling, the ether layer is carefully decanted into a 500 cc. Erlenmeyer flask, care being taken that none of the watery layer passes over into the flask. Three ether extractions are made. The ether is then removed by distillation until only 5 cc. of ether remain. To the residue are added approximately 5 cc. of a 1:1 mixture of alcohol and acetone. This is warmed on a water bath until the volume is reduced to about 3 cc. and decanted into a 15 cc. centrifuge tube. The alcohol-acetone extraction is made three times until the volume of solution in the centrifuge tube is approximately 10 cc.

Free Cholesterol—A drop of phenol red (0.02 per cent) is added, and, if the extract is not acid, 5 per cent hydrochloric acid is added to make it slightly acid. 1 drop of a 0.7 per cent water solution of digitonin is added, producing a slight turbidity. The tubes are then placed in a water bath at 50°. When warm, to each tube are added, from a burette slowly and with slight stirring,

4 cc. of 0.7 per cent aqueous digitonin solution. The tube is returned to the bath to coagulate the fine precipitate. The mixture is allowed to stand at room temperature overnight. The next day the stirring rods are removed to a rack designed to hold them without removing any of the precipitate which may adhere to the rod. The tubes are then centrifuged. After centrifugation 4 drops of 0.5 per cent alcoholic solution of digitonin are added. If the supernatant fluid becomes cloudy, the cholesterol has not been completely precipitated and more digitonin must be added. If precipitation is complete, alcohol-acetone is added to 15 cc., and the entire precipitate stirred up and again centrifuged. Most of the supernatant fluid is removed by gentle suction. The stirring rods are then replaced in the same tubes from which they were removed and to the precipitate are added 10 cc. of alcohol-acetone; the precipitate is stirred up, the rods removed, and the mixture again centrifuged and the supernatant fluid removed. The precipitate is washed twice again, each time with 10 cc. of ether. The precipitate may then be allowed to stand at room temperature overnight, or dried in the oven at 37°. To insure complete dryness the tubes are heated in the oven for 10 minutes at 100° just before the color development. To the dry precipitate are added 3.0 cc. of acetic acid; the mixture is heated at 50–60° until the precipitate dissolves, cooled, and then 6.0 cc. of acetic anhydride are added. The solution is thoroughly mixed by stirring, and then 0.3 cc. of concentrated sulfuric acid is added, stirred in, and the tube allowed to stand 25 minutes for color development. At the end of this time the solutions are compared in the colorimeter with a suitable standard.

Total Cholesterol—A drop of phenol red and 0.1 cc. of 33 per cent potassium hydroxide are added to the alcohol-ether extract, mixed thoroughly, and the tube placed in the oven at 37–40° for 2 hours. After saponification, the mixture is made slightly acid with 5 per cent hydrochloric acid, and the total cholesterol determined in the same way as described under "Free cholesterol."

DISCUSSION

In Table I are given representative results of analyses of standard chloroform solutions of cholesterol and cholesterol acetate,

and in Table II representative results of analyses of bile and bile plus added cholesterol.

Sample of Bile—Suggested quantities are for dog hepatic bile 10 cc., for dog gallbladder bile 3 cc., for human hepatic bile 3 to 5 cc., and for human gallbladder bile 0.5 to 2 cc. The color development is most satisfactory for amounts of cholesterol between

TABLE I

Analysis of Standard Solutions

Free cholesterol, Merck's c.p.; ester cholesterol, Eastman's cholesterol acetate.

Experi-ment No.	Theoretical cholesterol			Cholesterol found		Per cent recovery	
	Free	Ester	Total	Free	Total	Free	Total
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
1	0.74	0.51	1.25	0.75		101	
2	1.47	1.02	2.49	1.50		102	
3	2.21	1.53	3.74	2.19		99	
4	3.68	2.55	6.23	3.74		102	
5	2.21	1.53	3.74		3.71		99
6	2.21	1.53	3.74		3.64		97
7	2.21	1.53	3.74		3.69		99
8	2.21	1.53	3.74		3.62		97
9	2.00		2.00	2.05		103	
10	2.00		2.00	2.04		102	
11	2.00		2.00		1.99		99
12	2.40		2.40	2.41		101	
13	2.40		2.40		2.40		100
14	2.14		2.14	2.13		99	
15	5.35		5.35	5.44		102	
16	1.36		1.36		1.36		100
17	0.68		0.68		0.69		102
18	2.71		2.71		2.78		103
19	6.78		6.78		6.50		96

0.5 and 5 mg. Amounts up to 8 mg. may be determined, but the bulk of the digitonide precipitate as well as the darkness of the color makes the determination less easy.

Magnesium Sulfate—This was added for two reasons: (1) it very frequently prevents formation of emulsions and produces a more rapid settling out of the two layers after shaking; (2) in certain specimens of bile it has been found that complete extrac-

tion of cholesterol with ether occurs more readily when magnesium sulfate is added.

TABLE II

Analysis of Dog Bile Plus Added Cholesterol

Experiment No.	Dog bile	Added cholesterol			Calculated		Found		Per cent recovery	
		Free	Ester	Total	Free	Total	Free	Total	Free	Total
	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
Mixed gallbladder and hepatic bile										
1	5						2.49			
2	5						2.44			
3	5							2.50		
4	5							2.55		
5	5							2.48		
6	5	1.47	1.02	2.49	3.94 (1.47 + 2.47)	4.99 (2.49 + 2.50)		5.14		103
7	5	1.47	1.02	2.49	3.94	4.99		4.95		99
8	5	1.47	1.02	2.49	3.94	4.99		4.97		99
9	5*	0.74	0.51	1.25	3.21 (0.74 + 2.47)	3.75 (1.25 + 2.50)		3.65		97
Hepatic bile										
10	10						1.76			
11	10						1.73			
12	10							1.91		
13	10							1.85		
14	10	0.74	0.51	1.25	2.48 (0.74 + 1.74)	3.15 (1.25 + 1.90)	2.44		98	
15	10	0.74	0.51	1.25	2.48	3.15	2.46		99	
16	10	0.74	0.51	1.25	2.48	3.15		3.19		101
17	10	0.74	0.51	1.25	2.48	3.15		3.15		100
18	10	1.47	1.02	2.49	3.21 (1.47 + 1.74)	4.39 (2.49 + 1.90)	3.14		97	
19	10	1.47	1.02	2.49	3.21	4.39		4.30		98

* Saponification with 20 per cent potassium hydroxide before ether extraction (as for Autenrieth-Funk analysis).

Ether Extraction—Table III shows the results of various extraction procedures on dog bile and on pathological human bile.

We have adopted the uniform procedure of extracting each sample three times, using 100 cc. of ether for each extraction. Extraction of the bile with ether gives a clear extract which at the most has only a slight yellow or green color. This eliminates working with a highly colored extract or residue,

TABLE III

Variations in Ether Extractions

Human bile Sample No.	Ether extraction	Found	Dog bile Sample No.	Ether extraction	Found
	cc.	mg. per 100 cc.		cc.	mg. per 100 cc.
Hepatic			Hepatic		
4	2 × 80	92.0	1	4 × 80	7.4
		95.7			8.2
	4 × 80	97.3		2 × 150	7.8
		96.3			7.6
8	2 × 80	83.0	2	2 × 100	13.1
		82.0			13.0
	4 × 80	83.7		3 × 100	13.0
		83.7			13.2
15	4 × 80	179.0		4 × 100	13.0
	2 × 100	165.0			13.1
	2 × 150	164.0	Gallbladder		
19	3 × 50	101.0	1	2 × 100	106.0
	4 × 80	106.0			101.0
	2 × 150	103.0		3 × 100	101.0
	3 × 100	105.0			102.0
Gallbladder				4 × 100	102.0
11	4 × 80	102.0			103.0
	2 × 150	82.0	2	2 × 80	.87.3
5	2 × 100	210.0			87.3
		213.0		4 × 80	89.0
	3 × 100	218.0			87.7
		218.0		8 × 80	91.0
	4 × 100	215.0			87.3
		217.0			

such as one obtains when the Schoenheimer-Sperry or the Wright (2) extraction procedure is used. The residue after ether evaporation is easy to handle and goes into solution in alcohol-acetone without any difficulty, and in this respect is superior to the residue obtained in the Wright procedure. Also any pigment

carried over into the digitonide precipitate will be removed with the subsequent ether washing.

Saponification—Numerous determinations were run with different amounts of potassium hydroxide and different times of saponification. The procedure of saponifying with 33 per cent potassium hydroxide for 2 hours at 37–40° is recommended as most satisfactory.

This procedure gives, as one would expect from previous observations of other workers, somewhat lower values than those obtained on the same specimens of bile by the Autenrieth-Funk procedure.

SUMMARY

A method for the determination in bile of 0.5 to 5 mg. of cholesterol (free and combined) has been described which is simple and accurate to ±5 per cent.

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THE EXCRETION OF KYNURENIC ACID BY THE MAMMALIAN ORGANISM. A METHOD FOR THE IDENTIFICATION OF SMALL AMOUNTS OF KYNURENIC ACID

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Kynurenic acid is a well established excretory product of both the dog and the rabbit. The compound has also been reported to have been found in the urine of the rat and coyote but not in that of the fox, wolf, cat, and man. Thus, the literature[1] on the subject is sufficient to indicate the possibility of an interesting correlation between zoological classification and the production of kynurenic acid. In this communication are presented further data on this question relative to the rat, cat, Dalmatian coach dog, and guinea pig.

Hopkins, commenting upon the rat, in a note appended to a paper by Asayama (1916) states: "The animal certainly excretes extremely little of the substance, but after feeding with tryptophane I have been able to separate small amounts of a substance from rats' urine which gives the reactions of kynurenic acid and melts at 88–89°."[2] Inasmuch as these data are quite limited, it

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* Alexander Brown Coxe Fellow, Yale University, 1934–35.

† Part of the data reported herewith is from a thesis submitted by Robert E. Kaufman in partial fulfilment of the requirements for the degree of Doctor of Medicine, Yale University, 1933.

[1] A résumé of the literature on kynurenic acid is given by Robson (1924).

[2] In a personal communication to one of us, Professor Hopkins states that the melting point reported in this note was undoubtedly a typographical error, the melting point presumably being 288–289°.

appeared desirable to study this species more extensively.   Mendel and Jackson (1898) were unable to find kynurenic acid in the urine of the cat subjected to a variety of dietary and metabolic conditions, including a high protein diet.[3]   However, the circumstances of its elimination by other species led us to consider that the cat might excrete kynurenic acid under the most propitious condition; *viz.*, that of flooding the body with tryptophane administered in pure form.   Experiments with the Dalmatian coach dog were undertaken because of its peculiar purine metabolism compared to that of other dogs.   It seemed barely possible that the Dalmatian dog might also exhibit a marked difference in the matter of kynurenic acid excretion.   Studies on the guinea pig in this connection have not been reported heretofore, so far as we are aware.

<div align="center">EXPERIMENTAL</div>

Our experiments required not only a method for isolating kynurenic acid from the urine but also a procedure for its certain identification.   The scheme of Capaldi (1897, *b*) is, as has been pointed out by several investigators, quite satisfactory for the isolation, but the product thus secured is obviously contaminated and can be freed of tenacious impurities only with much difficulty. Moreover, the melting point of pure kynurenic acid (nearly 300°) is, in fact, a decomposition point which depends upon the rate of heating.   Indeed, the reported values for the crude substance are so diverse that a more reliable criterion for the identity of the acid is needed.   Späth (1921) has shown that the fairly sharp melting point (224°) of the methyl ester may be employed as a physical constant for identification of the acid, and has suggested the preparation of this derivative from small amounts of material. We have confirmed Späth's experiment in which 25 gm. of kynurenic acid yielded 14 gm. (52 per cent) of the ester, and have further been able to adapt the procedure to amounts of 100 and even 50 mg. of the acid.   Consequently, for the final identification of kynurenic acid samples, we have employed the melting point of the methyl ester and the melting point of a mixture with an authentic analyzed specimen.[4]

[3] Kotake (1935) mentions that N. Iwakura and T. Kiyomatsu were not able to demonstrate the formation of kynurenic acid by the cat.

[4] The authentic specimen of kynurenic acid methyl ester was prepared

Preparation of Methyl Ester of Kynurenic Acid on a Small Scale
—100 mg.[5] of crude kynurenic acid, dried at 110°, are suspended in 3 cc. of absolute methyl alcohol in a 15 cc. centrifuge tube equipped with an inlet tube dipping into the fluid, and a small reflux condenser leading to a drying tube containing calcium chloride. The centrifuge tube is placed in a water bath at 60° and dry hydrogen chloride is bubbled in for 5 hours at this temperature, although undoubtedly the solution becomes saturated in a much shorter time. All of the kynurenic acid goes into solution at first, although sometimes a precipitate subsequently forms. In either case, after about 2½ hours, large crystals of the ester hydrochloride begin to appear. At the end of 5 hours, the water bath is removed and the flow of gas continued for another ½ hour. The inlet tube and the condenser are now removed and the former washed down with a minimal quantity (about 1 cc.) of absolute methyl alcohol saturated with dry hydrogen chloride. The centrifuge tube is now stoppered and placed in the ice box overnight. The crystalline precipitate is centrifuged and washed twice with small portions (0.5 to 1 cc.) of cold absolute methyl alcohol saturated with dry hydrogen chloride. The crystals remaining in the centrifuge tube are dissolved in a minimal quantity of hot water (about 0.5 cc.), centrifuged at once, and the clear solution decanted from a small insoluble residue. The solution (but not the residue) remaining in the tube is rinsed out with a few drops of water. This solution is then cooled in ice and made alkaline to litmus by the addition of a saturated solution of potassium bicar-

from natural kynurenic acid. The ester was light yellow in color and melted at 224-225° (corrected). $C_{11}H_9NO_3$: calculated, C 65.00, H 4.47, N 6.90; found, C 65.05, H 4.56, N 6.75. The identity of the substance was further verified by comparison with the ester secured from a synthetic specimen of kynurenic acid supplied by Hoffmann-La Roche, Inc. 100 mg. of the synthetic acid, prepared according to the German patent No. 575,534 (*cf. Chem. Abst.*, **27**, 4816 (1933)) by saponifying the product produced by heating oxalacetic ester anil, yielded a first crop of 52 mg. of pure white crystals of the methyl ester melting at 225°. The melting point of a mixture of this ester and the reference specimen described above was not depressed.

[5] Esterifications on 50 mg. batches were run in exactly the same way as those on 100 mg. samples except that 1.5 cc. of absolute methyl alcohol instead of 3 cc. were employed in the esterification. Subsequently, the procedure was the same in both instances.

bonate. Inasmuch as the free ester may separate from solution at once or only after standing 30 minutes to 1 hour in an ice-salt bath, the solution is regularly allowed to stand in the bath for 1 hour. Vigorous stirring of the solution and scratching of the container may be necessary to induce crystallization; seeding also helps. The resulting precipitate is filtered off, dried at 110°, and weighed. The product is somewhat more colored than that prepared on a large scale.

The original methyl alcohol mother liquor and washings evaporated to dryness in the vacuum desiccator over potassium hydroxide generally yield a brown semicrystalline residue. The material is transferred to a small centrifuge tube with the aid of cold absolute methyl alcohol saturated with dry hydrogen chloride, centrifuged, and the precipitate is then washed with two small portions of the same solvent. The ester is liberated from its hydrochloride as in the first instance.

The size of this second crop of material in various runs varies with the purity of the original kynurenic acid. In the case of pure acid, only a small amount of ester was obtained in the second crop. For example, duplicate esterifications of 50 mg. of the pure acid[6] by the above procedure yielded first crops of the ester of 22 and 23 mg. and a second crop in each case of 7 mg. All of these crops were colorless and melted at 224°.[7] In the case of the crude acids, the total yields were smaller, with a greater proportion of the material in the second crop. In one instance, there was no first crop of crystals. The yields of ester from the crude acids are illustrated in the experiments reported below.

Experiments on the Albino Rat—Male rats weighing from 200 to 300 gm. were employed. A basal diet consisting of casein 18, dextrin 48, Osborne and Mendel (1919) salt mixture 4, dried yeast 5, lard 20, and butter 5 per cent was given *ad libitum*. Tryptophane was administered orally by incorporating it in 10 gm. (generally) of the basal diet. At the beginning of the experiment this mixture replaced the basal diet until the mixture was entirely

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[6] The colorless, crystalline acid was prepared in practically quantitative yield from the authentic specimen of the methyl ester and was found to melt with fairly rapid heating at about 290°. Analysis of the acid: N calculated, 7.41; N found, 7.46 per cent.

[7] All melting points are corrected.

consumed.   A modified Hopkins type of metabolism cage was
employed to collect the urine, which was preserved with toluene.
At the end of the experiment the funnels and cage bottoms were
washed down with boiling water or with dilute ammonia solution.
The urines were collected for 2 to 4 days and in every case for at
least 1 day after the last tryptophane was given.   The urines
diluted with the wash water were treated according to the proce-
dure of Capaldi, and any kynurenic acid secured was identified
by the method previously outlined.   The results presented in

TABLE I

Excretion of Kynurenic Acid by the Rat

Animal No.	Dose of trypto-phane (*per os*)	Yield of kynurenic acid*	Identification†				
			Amount of acid esterified	Ester			
				Crop 1		Crop 2	
				Yield	M.p.	Yield	M.p.
	gm.	*mg.*	*mg.*	*mg.*	*°C.*	*mg.*	*°C.*
1	2.0	267	100	None		20	213
2	1.0	172	100	20	221	13	219
3	1.0	187	100	30	220	19	218
3	2.0	322	100	35	221	9	220
2	1.0‡	70	50	17	222	3	221

   * The melting points of the kynurenic acid samples (isolated by the
Capaldi procedure) reported in both Tables I and III lay between 265–275°.
   † All samples of kynurenic acid methyl ester described in this paper were
mixed with the authentic specimen of the ester and melting points of the
mixtures were taken.   Since all these melting points lay between that of
the reference material and the respective melting points of the samples
to be identified, the melting points of the mixtures are not recorded.
   ‡ This was given by subcutaneous injection.

Table I show that the rat excretes appreciable amounts of ky-
nurenic acid.
   Several control urines were collected under conditions exactly
similar to those described above, except that no tryptophane was
administered to the animals.   These urines, subjected to the
Capaldi method, yielded either no precipitates or only a trace of
tarry material.
   Experiments on the Cat—The cats were confined in metal me-
tabolism cages and given a liberal diet of milk.   Tryptophane was

administered by subcutaneous injection. For this purpose, the amino acid was ground to a fine powder and suspended in 35 to 45 cc. of water warmed to body temperature and the injection made with a No. 15 needle. The tryptophane remaining in the syringe was suspended again in a little more water and the injection completed. It was a rather common occurrence for the cats to vomit following these injections. When this happened, the cage was changed in order that the urine would not be contaminated. The urines were preserved with toluene. The results of the Capaldi procedure applied to these urines are shown in Table II.

TABLE II

Experiments on Excretion of Kynurenic Acid by the Cat

Animal No.	Sex	Weight	Dose of tryptophane (subcutaneously)	Period of urine collection	Volume of urine	Weight of precipitate by Capaldi method
		kg.	gm.	hrs.	cc.	mg.
1	F.	3.6	None	72	735	0.6
1	"	3.6	3.00	70	770	4.6
2	"	2.1	3.85	72	360	3.6
3	M.	2.1	3.85	24	120	2.5
4	F.	2.2	4.00	46	520	4.5
5	"	2.0	None	66	700	None
5	"	2.0	3.50	48	600	0.7

The precipitates obtained were in every case highly pigmented, of a tarry nature, and did not possess the characteristic appearance of kynurenic acid. Moreover, a precipitate was obtained at times when no extra tryptophane was given. Indeed, the largest amount of precipitate calculated as kynurenic acid would represent a yield from the tryptophane administered of only 0.1 per cent. The efficacy of the Capaldi procedure applied to cat urine was demonstrated by recovering practically quantitatively known added amounts of kynurenic acid.

Is Kynurenic Acid Destroyed by the Cat?—Inasmuch as all efforts to demonstrate the production of kynurenic acid in the cat were unsuccessful, the question arose as to whether the acid was synthesized and then as an intermediate completely destroyed. The following experiments were planned to supply the answer. A

female cat weighing 2.2 kilos was maintained on a diet of 125 gm. of chopped meat per day and injected subcutaneously with 300 mg. of pure kynurenic acid.[6] For injection, the acid was dissolved in about 7 cc. of water with the aid of sodium bicarbonate, the carbon dioxide driven off, and the reaction adjusted to about pH 7.5. The urine collected for 72 hours amounted to 290 cc. and yielded by the Capaldi method 208 mg. of kynurenic acid melting at 274°. In a second experiment with the same animal, 500 mg. of the acid were injected in like manner. The urine collected for 48 hours amounted to 145 cc. and yielded 459 mg. (92 per cent recovery) of kynurenic acid melting at 275°. The urine collected for the next 48 hours yielded none of the product.

These recoveries of kynurenic acid, especially that in the second experiment, make it appear highly unlikely that the cat converts any appreciable amount of a 4 gm. dose of tryptophane (cf. Table II) to kynurenic acid and then destroys the latter. It, therefore, seems improbable that kynurenic acid is an intermediate in the catabolism of tryptophane by the cat. The dog and rabbit have been reported in some experiments likewise to excrete injected kynurenic acid practically quantitatively (for the results of various authors, consult Kotake and Ichihara (1931)). In similar experiments upon man, a recovery of injected kynurenic acid as high as 70 per cent has been claimed (Solomin, 1897). It is to be noted that of these four species, the cat and apparently man do not excrete kynurenic acid, while the dog and the rabbit do excrete the compound. It is still a question whether the cat produces and excretes kynurenine (cf. Kotake and Iwao (1931)) in the degradation of tryptophane.

Experiment on the Dalmatian Coach Dog—A female Dalmatian dog,[8] very fat and weighing about 30 kilos, was given 5 gm. of tryptophane orally in eight No. 00 gelatin capsules. The amino acid was given in two equal doses at a 2 hour interval. Chopped lean meat was fed throughout the experiment, beginning 1 day before the administration of tryptophane. 1 kilo of the meat was consumed during the period of urine collection. Urine amounting to 770 cc. was secured from the metabolism cage during a 46 hour period. The yield of kynurenic acid isolated from the total urine by the Capaldi method was 1.73 gm. The acid melted at 277°.

100 mg. yielded 60 mg. of the methyl ester, m.p. of 223°.   It is obvious that the Dalmatian dog like other dogs excretes kynurenic acid.

Experiments with the Guinea Pig—The guinea pigs, all females, were placed in the same type of cage employed for the rats and given a mixed diet of oats, carrots, lettuce, cabbage, and water.   The tryptophane was administered by subcutaneous injection as in the case of the cat experiments.   The results of the various tests are shown in Table III.   The yields of ester from the kynurenic acid secured in these experiments are rather poor but the data nevertheless show conclusively that the guinea pig excretes kynurenic acid.

TABLE III

Excretion of Kynurenic Acid by the Guinea Pig

Animal No.	Weight	Dose of tryptophane (subcutaneously)	Period of urine collection	Yield of kynurenic acid	Identification				
					Amount of acid esterified	Ester			
						Crop 1		Crop 2	
						Yield	M.p.	Yield	M.p.
	gm.	*gm.*	*hrs.*	*mg.*	*mg.*	*mg.*	*°C.*	*mg.*	*°C.*
1	640	None	78	None					
2	760	"	44	"					
3	490	"	72	"					
1	500	1.50	27	129	100	15	221	13	220
2	650	1.50	48	74	50	7	221	4	220
3	540	1.50	48	54	45	13	221	1	

DISCUSSION

A summary of the original literature bearing on the excretion of kynurenic acid by various species is given in Table IV.   It is noteworthy that all three members of the rodent family so far studied eliminate the compound.   The findings reported by Capaldi (1897, *a*) for the fox and wolf are negative, but inasmuch as two other species in the same family are known to excrete kynurenic acid, and inasmuch as no experiments involving the administration of tryptophane *per se* to the fox and the wolf have been recorded, we consider it possible that these animals under more favorable conditions may excrete the product.   In support

of this contention may be cited experiments with several species showing that there is a minimum amount of tryptophane which must be administered to the animal within a given period in order that the excretion of kynurenic acid will be demonstrable. It is to be noted that we have shown by comparison of the methyl ester derivatives that the various samples of kynurenic acid from the dog (Dalmatian dog), rat, guinea pig, and rabbit (data not reported) are all one and the same compound.

TABLE IV

Summary of Data on Excretion of Kynurenic Acid by Various Members of the Class Mammalia

Order	Family	Common name	Excretion of kynurenic acid	References
Rodentia	Leporidæ	Rabbit	+	Ellinger (1904)
	Muridæ	Rat*	+	Hopkins (1916); authors
	Caviidæ	Guinea pig	+	Authors
Carnivora	Canidæ	Dog	+	Liebig (1853)
		Dalmatian dog	+	Authors
		Coyote†	+	Swain (1905)
		Fox	−(?)	Capaldi (1897, *a*)
		Wolf	−(?)	" (1897, *a*)
	Felidæ	Cat	−	Mendel and Jackson (1898); authors
Primates	Hominidæ	Man	−	Hofmeister (1881); Ellinger (1904)

* *Mus norwegicus albinus.*

† *Canis ochropus*, Eschscholtz (*cf.* Swain, 1905).

SUMMARY

1. A method for the identification of small amounts of kynurenic acid as the methyl ester is described.

2. It has been shown that the rat, guinea pig, and Dalmatian dog excrete kynurenic acid and that the cat does not excrete this product, even following the administration of large amounts of tryptophane.

3. Experiments showing that the cat does not destroy kynurenic acid are reported.

4. A summary of the data on the excretion of kynurenic acid by various species is presented.

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DOES BIS(2-AMINOETHYL) DISULFIDE (CYSTAMINE) PROMOTE GROWTH IN THE RAT LIMITED TO AN INADEQUATE INTAKE OF CYSTINE AND METHIONINE?*

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Cystamine[1] has been reported by Sullivan, Hess, and Sebrell (1931) to promote growth in the white rat subjected to a nutritional deficiency of cystine. Such a replacement would be of considerable interest inasmuch as cystamine would then be shown to be the only sulfur derivative,[2] differing in its length of carbon chain from both cystine and methionine,[3] known to replace these amino acids in the diet. The above authors based their conclusion on the finding that the average daily gain in body weight of four animals receiving cystamine hydrochloride (comprising 0.5 per cent of the diet) during a period of 20 weeks was 0.3 gm. greater than the average for four control animals on the basal diet alone.

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[1] We have used the name cystamine as employed by Robbers (1934) rather than cystine amine suggested by Sullivan, Hess, and Sebrell (1931). The name cystamine conforms to the customary usage, as, for example, tyramine, tryptamine, etc.

[2] The claim of Mitchell (1924) that taurine can serve as a dietary substitute for cystine could not be confirmed by Beard (1925–26), Lewis and Lewis (1926), or by Rose and Huddlestun (1926).

[3] The deficiency is discussed as one of both cystine and methionine because, as discovered by Jackson and Block (1931) and confirmed by Weichselbaum, Weichselbaum, and Stewart (1932), and others, either of these amino acids promotes growth of the rat on the various diets that have been employed to develop the so called cystine deficiency.

In view of our repeated observation of very considerable differences in the individual growth responses of animals to each of several low cystine-methionine diets, we were inclined to hold that this evidence was not completely convincing. Furthermore, Sullivan, Hess, and Sebrell employed separate and distinct litters of animals to compare the growth effects of the basal diet alone and the basal diet with added cystamine. The authors state, "The cystine amine experiment was carried on later with a different set of litter mates." This was done, notwithstanding that it has been shown that different litters *may* exhibit appreciable variation in growth upon the type of deficient diet in question.[4] The authors did not report food consumption.

We therefore undertook a reexamination of the availability of cystamine for growth and published a note (Block and Jackson, 1932) to the effect that we could find no basis for the claim of Sullivan, Hess, and Sebrell. Inasmuch as their conclusion has since been quoted without qualification in one text-book (Harrow and Sherwin, 1935) as well as elsewhere in the literature (Robbers, 1934), we are herewith submitting our detailed findings.

EXPERIMENTAL

The cystamine was synthesized by condensing potassium phthalimide with 1 mole of ethylene dibromide, treating the product with potassium hydrogen sulfide, oxidizing the mercaptan to the disulfide, and finally hydrolyzing with hydrochloric acid in a bomb to give phthalic acid and cystamine, essentially according to the directions of Gabriel (1891) and of Coblentz and Gabriel (1891). 10 gm. of bis(2-phthalimidoethyl) disulfide melting at 136–137°[5] thus yielded 5.8 gm. of crude cystamine hydrochloride after the removal of the most of the phthalic acid. The product

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[4] The litter factor in its relation to growth of the rat on cystine-deficient diets is discussed by Sherman and Woods (1925) and Lewis and Lewis (1926). It is obvious that caution is necessary in comparing the growth of different litters on the type of diet in question. Chart I in the paper by Rose and Huddlestun (1926) is cited further to bring out the point. Over a period of about 13 weeks, the difference in the average individual daily growth of two litters on the basal diet or the basal diet with added taurine (which has been found not to influence growth) amounted to about 0.5 gm. per day. This difference is obviously without particular import and yet is greater than the 0.3 gm. difference to which significance is ascribed by Sullivan, Hess, and Sebrell.

[5] All melting points are corrected.

was recrystallized from 95 per cent alcohol to give slightly more than 4 gm. of a colorless substance melting at 212°, and this in turn was recrystallized to give 2 gm. of beautiful plates (not needles) melting at 215° (213–214° with a somewhat slower rate of heating of 1° per minute).

Analysis

$C_4H_{12}N_2S_2 \cdot 2HCl$.	Calculated.	C 21.32,	H 6.27,	N 12.44,	Cl 31.50,	S 28.49
	Found.	" 21.77,	" 6.21,	" 12.05,	" 31.30,	" 28.71

The substance was, of course, extremely soluble in water and following reduction with sodium cyanide gave a magenta color with a solution of sodium nitroprusside. This specimen of material was used in the feeding tests described below.

Male albino rats were employed as test animals. At weights of about 60 to 75 gm., they were transferred from the regular stock diet to the basal diet (Diet B), low in cystine and methionine as shown in previous studies. This diet was composed as follows: whole milk powder 15, gelatin 2, salt mixture (Osborne and Mendel, 1919) 1, sodium chloride 1.7, corn-starch 54.7, Lloyd's reagent adsorbate of vitamin B[*] 0.6, and lard 25 per cent. 100 mg. of cod liver oil and 125 mg. of dried yeast were given separately daily to each rat. Our technique was to select for experiment those animals whose body weights were most nearly stationary. After the growth behavior of the animal on the basal diet had been clearly demonstrated, the diet was supplemented with cystamine hydrochloride for a period of sufficient length to demonstrate whether this substance exerted any effect on growth, and finally, either with or without another period of growth on the basal diet alone, cystine was incorporated in the diet to show that the animal still possessed the capacity to grow vigorously at the end of the experiment. Thus each experiment on any animal was controlled by the growth behavior of the same animal both on the basal diet and on the basal diet supplemented with cystine. A more detailed discussion of the advantages of this method has been presented in an earlier communication (Jackson and Block, 1932). 120 mg. of l-cystine (arbitrarily called 1 equivalent) and 450 mg. of cystamine hydrochloride (4 equivalents) were employed as supplements for 100 gm. of the basal diet. The purity of the cystine was established by nitrogen determination.

[*] This concentrate was particularly rich in vitamin B(B_1). It was kindly supplied by Eli Lilly and Company, Indianapolis.

Results

Three of our five experiments, all of which are consistent in showing that cystamine does not substitute nutritionally for cystine and methionine, are illustrated in Chart I. The three animals whose growth graphs are depicted were from three separate litters. It is to be observed that the introduction of cystamine
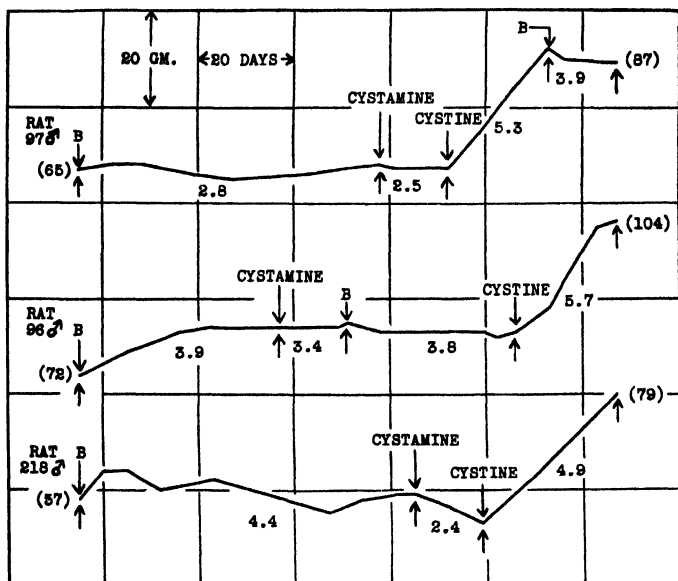

CHART I. Growth on the cystine-deficient Diet B and on Diet B supplemented with cystamine (4 equivalents of the hydrochloride; see text) and with *l*-cystine (1 equivalent). The introduction of each diet is indicated by name or symbol over a downward arrow. The average daily food consumption in gm. is shown by a number inserted between two upward arrows demarking the period in question. The initial and final body weights in gm. are printed within parentheses.

into the diet is in no case followed by any appreciable increase in body weight. The over-all weight changes during the 2 weeks of the ingestion of 4 equivalents of cystamine hydrochloride were, respectively, -1, $+1$, and -6 gm., whereas during the first 2 weeks of ingestion of 1 equivalent of cystine, the corresponding weight changes were $+18$, $+16$, and $+13$ gm.

In addition to our preceding comments on the technique em-

ployed by Sullivan, Hess, and Sebrell, it may be noted that in regard to any differences in experimental conditions (diet, etc.) employed in the two studies, the animals used in our tests exhibited a more pronounced restriction of growth. It is therefore only logical to assume that our animals would have more readily revealed any *real* stimulation of growth brought about by a supplement of cystamine. *We therefore conclude that cystamine*[7] *is devoid of growth-promoting properties under the stated conditions.*

The authors are indebted to Mr. William Saschek for some of the analyses and to Miss Doris Blumenthal for aid in the preparation of some of the chemical intermediates.

Addendum—After the preparation of this report had been completed, there appeared a communication on the same subject by Mitchell (1935) who concludes, "No evidence has been obtained that cystine amine can perform the functions of cystine in promoting animal growth." This is a confirmation of our conclusion, previously reported to the American Society of Biological Chemists (Block and Jackson, 1932), from the investigation presented in detail herewith.

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[7] The effect of 2-aminoethanethiol (cysteamine) on growth has apparently not been investigated.

THE TRUE BLOOD SUGAR LEVEL IN INSULIN SHOCK AND CONVULSIONS

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In previous work done on the blood sugar level of insulinized animals, attempts have been made to determine the blood sugar level during convulsions. The results have been very inconsistent until Dotti (1), working with rabbits, found that at the incidence of convulsions the true blood sugar level is zero.

Since the rat manifests all stages of insulin reaction ranging from simple weakness to convulsions, it was thought worth while to determine the true blood sugar level of rats at the incidence of convulsions as well as during the time of marked prostration which precedes the onset of the convulsions. The rabbit exhibits a short period of prostration with sudden onset of convulsions, while the rat passes through a prolonged period of weakness before convulsions become manifest. In the rabbit this period of weakness lasts but a few minutes, while in the rat it may last for an hour or more.

All experiments were performed upon rats which had been fasted for 24 hours. 10 units of Lilly's insulin were injected per kilo, subcutaneously, and at the appropriate time, as indicated by the symptoms, the animals were killed by decapitation. The blood was collected in a small porcelain dish which had been dusted with powdered sodium citrate.

The blood proteins were precipitated by the method of Folin and Wu (2), and the reducing substances in the filtrate were determined by the Shaffer-Hartmann method (3). The conversion table of Duggan and Scott (4) was used for determining the reducing substance of the sample in terms of glucose. The fermentable fraction was removed by the yeast method of Somogyi (5). The

same precautions were taken with the yeast cells as were described in a previous paper (1). The results obtained are shown in Table I.

Dotti (6) has followed the blood sugar level of rabbits for 6 hours after 2 units of insulin per kilo. He found that the total blood sugar dropped progressively until the onset of convulsions. When convulsions did not occur, the blood sugar fell to a minimum value of about 50 mg. of total sugar, after which there was a recovery with a rising sugar level. Hrubetz (7), using subcon-

TABLE I

Reducing Power of Blood during Shock and at Convulsions

	Mean value	Mean deviation	Mean deviation of mean
Controls (50 observations)	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Total reducing substance........	114	12.0	1.7
Non-fermentable reducing substance........................	43	4.4	0.6
Fermentable reducing substance (by difference).................	71	11.0	1.6
Rats in shock (62 observations)			
Total reducing substance........	54	6.6	0.8
Non-fermentable reducing substance........................	38	6.6	0.8
Fermentable reducing substance (by difference).................	16	7.0	0.9
Rats in convulsions (53 observations)			
Total reducing substance........	42	5.0	0.7
Non-fermentable reducing substance........................	40	5.0	0.7

vulsive doses on rats, showed a progressive fall in the total reducing power through the 1st hour after injection, after which there was a return to the normal level. Dotti (1) has shown with rabbits that the non-fermentable fraction of the blood sugar does not change, at least not significantly, after insulin. The material presented in this paper shows that this is also true for rats. From these results it seems, therefore, that during the course of insulin shock, there is a progressive fall in the true blood sugar level through the period of prostration to the incidence of convulsions when this level is zero.

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THE IONIZATION OF LACTIC ACID FROM ZERO TO FIFTY DEGREES*

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Information concerning the changes in heat and free energy accompanying each biochemical reaction is necessary for the interpretation of metabolic experiments. These quantities may be determined by the use of precise physicochemical methods. In the present study, measurements have been made over an extended temperature range of the electromotive force of cells without liquid junctions containing lactate buffers. From these measurements, the ionization constant of lactic acid and the change in free energy, heat content, and entropy accompanying the ionization of lactic acid have been determined.

Preparation of Materials and Results

Although lactic acid has recently been prepared in a state of high purity (4), its purification is a matter of some difficulty. Since a number of the crystalline salts of this acid are well characterized and readily purified, they were selected as more suitable starting materials for the present work. Lithium, barium, strontium, and zinc lactates were made by the neutralization of c.p. lactic acid with the respective carbonates and recrystallized four times, the lithium and barium salts from alcohol-water mixtures. Krauskopf and Carter (10) found it difficult to crystallize barium lactate, but crystallization takes place readily from alcohol-water

mixtures.   Calcium lactate, U.S.P., was recrystallized seven times. The dried salts were analyzed by ignition and conversion to the sulfates, with the exception of the zinc salt, which was weighed as the oxide.   The analyses indicated impurities of less than 0.1 per cent.   Some of the salts were optically active, but lack of data on the pure optical isomers prevented an estimate of the
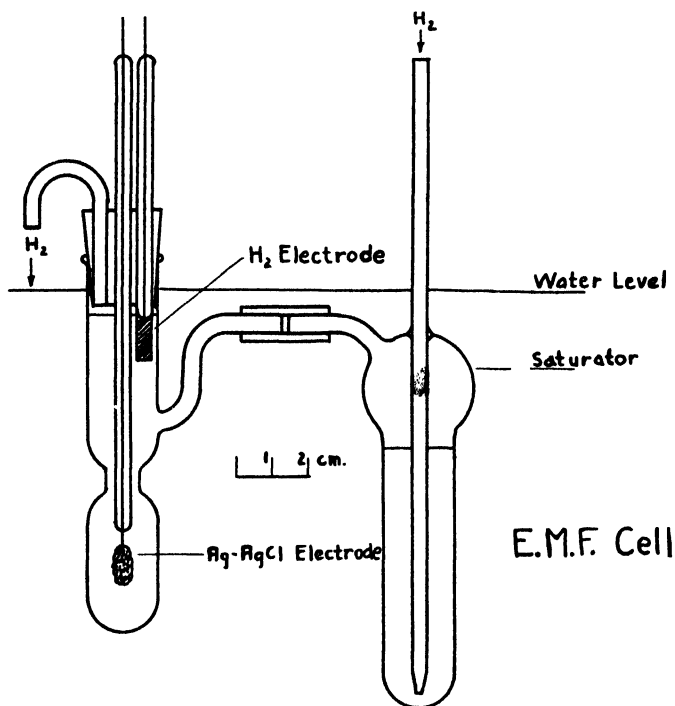

FIG. 1. The cell used in determining the ionization constant of lactic acid

amount of each present.   Hydrochloric acid was analyzed as silver chloride.   The preparation of the electrodes and solutions and the experimental details are similar to those described for ala-nine (13), with the exception that a refrigerated thermostat was used for the lower temperatures.

To make this method generally useful in biochemical work, it was necessary to modify it for measurements with smaller quanti-

TABLE I

Electromotive Force of Cells Containing Lactate Buffers

Lactate series	m_1	m_2	Observed E, corrected to 1 atmosphere H_2, at				
			0.3°	12.5°	25.0°	37.5°	50.0°
Lithium	0.002367	0.004724	0.59215	0.60050	0.60906	0.61743	0.62547
	0.005774	0.01152	0.56986	0.57727	0.58468	0.59194	0.59899
	0.009504	0.01902	0.55756	0.56438	0.57119	0.57792	0.58451
	0.01329	0.02659	0.54942	0.55579	0.56223	0.56865	0.57501
	0.01831	0.03653	0.54162	0.54774	0.55381	0.55982	0.56559
	0.02126	0.04255	0.53797	0.54391	0.54983	0.55566	0.56137
	0.02804	0.05611	0.53121	0.53688	0.54254	0.54808	0.55359
	0.03844	0.07693	0.52351	0.52884	0.53416	0.53934	0.54437
	0.04827	0.09660	0.51791	0.52299	0.52803	0.53294	0.53788
Barium	0.003134	0.003144	0.58535	0.59342	0.60153	0.60952	0.61741
	0.004264	0.004278	0.57737	0.58509	0.59282	0.60050	0.60809
	0.006435	0.006456	0.56710	0.57437	0.58155	0.58864	0.59557
	0.009504	0.009535	0.55741	0.56425	0.57110	0.57778	0.58435
	0.01313	0.01318	0.54947	0.55595	0.56239	0.56869	0.57488
	0.01594	0.01599	0.54456	0.55085	0.55709	0.56318	0.56920
	0.01931	0.01938	0.53988	0.54596	0.55199	0.55788	0.56365
	0.02592	0.02601	0.53248	0.53822	0.54394	0.54946	0.55483
	0.03275	0.03286	0.52679	0.53224	0.53766	0.54293	0.54811
Strontium	0.002645	0.002640	0.58913	0.59731	0.60553	0.61384	0.62179
	0.003004	0.002998	0.58576	0.59389	0.60207	0.61016	0.61798
	0.003582	0.003575	0.58116	0.58913	0.59708	0.60489	0.61254
	0.006482	0.006470	0.56618	0.57341	0.58063	0.58769	0.59468
	0.009768	0.009748	0.55581	0.56267	0.56946	0.57612	0.58259
	0.01303	0.01301	0.54857	0.55509	0.56156	0.56789	0.57410
	0.01676	0.01672	0.54223	0.54845	0.55466	0.56068	0.56652
	0.01926	0.01922	0.53872	0.54483	0.55084	0.55667	0.56236
	0.02443	0.02438	0.53254	0.53844	0.54423	0.54988	0.55531
Calcium	0.002489	0.002611	0.59170	0.60031	0.60888	0.61730	0.62539
	0.003169	0.003324	0.58542	0.59370	0.60198	0.61004	0.61778
	0.003513	0.003685	0.58273	0.59093	0.59905	0.60707	0.61468
	0.003824	0.004011	0.58074	0.58881	0.59683	0.60486	0.61256
	0.006779	0.007111	0.56540	0.57287	0.58023	0.58740	0.59439
	0.009879	0.01036	0.55541	0.56246	0.56941	0.57622	0.58274
	0.01656	0.01737	0.54139	0.54798	0.55443	0.56067	0.56658
	0.02364	0.02482	0.53157	0.53772	0.54385	0.54970	0.55515
	0.03049	0.03198	0.52442	0.53043	0.53621	0.54175	0.54694
Zinc	0.003175	0.003175	0.57898	0.58708	0.59505	0.60278	0.61026
	0.004206	0.004206	0.57032	0.57802	0.58543	0.59256	0.59939
	0.006462	0.006462	0.55718	0.56443	0.57151	0.57822	0.58459
	0.007391	0.007391	0.55297	0.56010	0.56693	0.57349	0.57977
	0.009531	0.009531	0.54494	0.55170	0.55824	0.56451	0.57042
	0.01291	0.01291	0.53521	0.54162	0.54774	0.55354	0.55897
	0.01589	0.01589	0.52847	0.53467	0.54054	0.54609	0.55127
	0.01739	0.01739	0.52562	0.53173	0.53753	0.54298	0.54813
	0.02857	0.02857	0.50906	0.51456	0.51974	0.52456	0.52904

ties of material. A cell (Fig. 1) was designed which holds less than 10 cc. of solution, thus effecting a considerable reduction in the total volume of solution required. The constriction in the cell prevents transfer of silver ions from the silver chloride electrode to the hydrogen electrode. The potentials observed with lactate solutions in the small cell agreed with those obtained from a larger H-type cell. Table I is a compilation of all the observed potentials.

TABLE II

Summary of E_0 (7) Used in Calculations, Values for pK Derived from Measurements, and Magnitude of Certain Thermodynamic Functions for the Process, Ionization of Lactic Acid

	0.3°	12.5°	25°	37.5°	50°
E_0 (7).....................	0.23618	0.22993	0.22239	0.21383	0.20436
pK (lithium series)........	3.891	3.867	3.862	3.874	3.896
" (barium series)........	3.892	3.868	3.864	3.876	3.898
" (strontium series).....	3.890	3.865	3.860	3.873	3.895
" (calcium series)	3.888	3.865	3.862	3.874	3.896
" (average)..............	3.890	3.866	3.862	3.874	3.896
" (from Equation 2).....	3.889	3.868	3.862	3.872	3.897
$\Delta F°$.....................	4865	5054	5267	5502	5760
$\Delta H°$.....................	795	460	−60	−620	−1265
$\Delta S°$.....................	−14.90	−16.25	−17.85	−19.70	−21.75

The electromotive force of the cell

$$\text{Pt, } H_2 \mid HCl(m_1) \text{ lactate}(m_2) \mid AgCl \mid Ag$$

where m_1 is the molality of hydrochloric acid, and m_2 is the molality of the metal lactate, is related to the ionization constant of lactic acid by the equation

$$pK - \log \frac{\gamma_{H^+} \gamma_{Cl^-} \gamma_{HL}}{\gamma_{H^+} \gamma_{L^-}} \quad \frac{(E - E_0)F}{2.3026RT} + \log \frac{m_1 (m_1 - m_{H^+})}{\nu m_2 - m_1 + m_{H^+}} \quad (1)$$

where ν is the valence of the cation of the salt, and L represents the lactate radical. The other terms have their usual significance. The numerical values of the fixed constants are taken from Birge's (2) compilation. The derivation of this equation is similar to others already described (6, 13). All the quantities on the right-

hand side of Equation 1 are known except the molality of the hydrogen ion. This may be estimated with more than sufficient accuracy by Equation 16 of the earlier communication (13). If the right-hand side of Equation 1 is plotted against the ionic strength, the intercept at 0 ionic strength is the negative logarithm of the thermodynamic ionization constant. The values of pK obtained by independent extrapolations of each series for all temperatures are listed in Table II.

DISCUSSION

Fig. 2 illustrates the curves obtained from the results at 25°. It is readily seen that the intercept at 0 ionic strength is defined to within ±0.003 pK unit. However the zinc series does not permit accurate extrapolation. The different slopes obtained are due to specific effects of the cation on the activity coefficient ratio, which appears on the left-hand side of Equation 1, if the salts present are assumed completely dissociated and no hydration takes place. This specific ion effect persists to the lowest measurable concentrations.

Harned and Embree (9) found that the variation with temperature of the ionization constants of many weak acids could be represented by a simple equation. For lactic acid the equation becomes

$$pK = pK_{max.} + 5 \times 10^{-5} (t - \theta)^2 = 3.862 + 5 \times 10^{-5} (t - 23.5)^2 \quad (2)$$

where θ is the temperature of maximum ionization. Fig. 3 shows the variation of pK with temperature. The agreement between Equation 2, which is represented by the curve, and the present results is excellent. A few determinations made by other investigators at isolated temperatures are also plotted.

Lactic acid is intermediate in strength between alanine (13) and propionic acid (8). This is to be expected as can be seen from the following considerations. The positive charge upon the amino group of alanine (postulated for solutions of low pH by the zwitter ion hypothesis) should repel the hydrogen of the carboxyl group with a greater force than an unchanged hydrogen atom or hydroxyl group; therefore alanine is the strongest acid. Also the replacement of an α hydrogen atom in propionic acid by a hydroxyl group should enhance the ease of ionization.
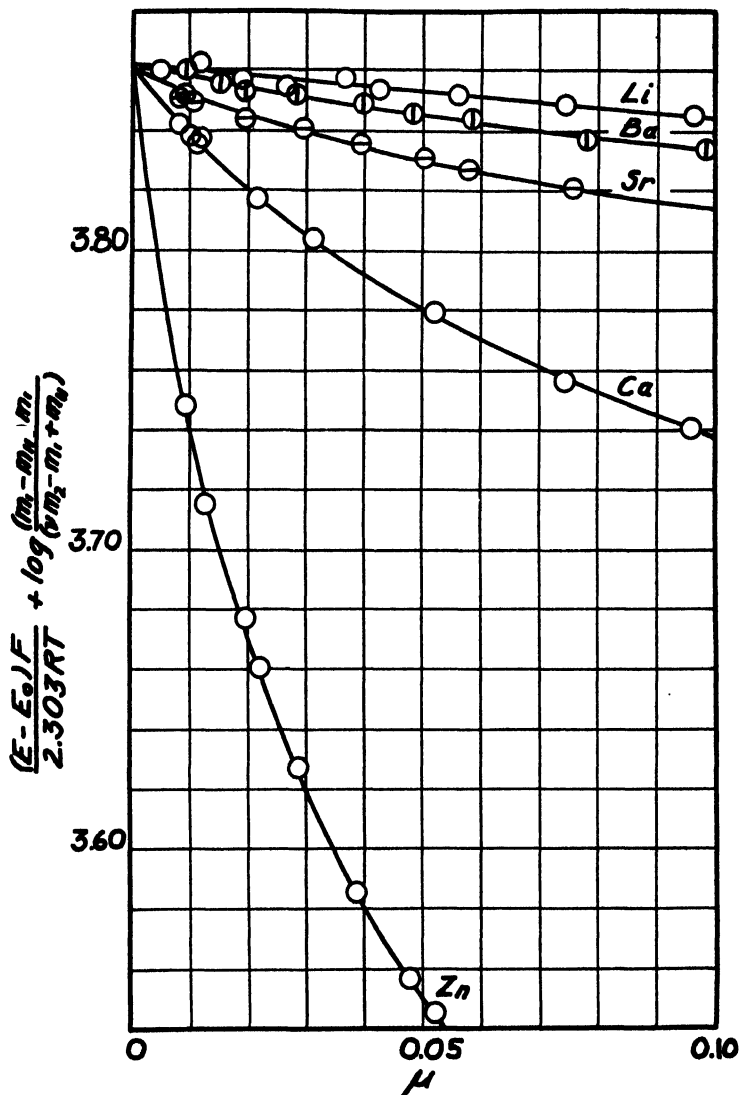
Fig. 2. A plot of the right side of Equation 1 against the ionic strength. The intercept at 0 ionic strength is the negative logarithm of the ionization constant.

The increase in free energy, heat content, and entropy due to the ionization process may be calculated for lactic acid by employing Equation 2 in conjunction with the proper thermodynamic definitions.

$$\Delta F^\circ \quad - RT \ln K = 2.3026 \, RT \mathrm{pK} \tag{3}$$

$$\Delta H^\circ \quad RT^2 \frac{d \ln K}{dT} = - 2.3026 \times 10^{-4} \, RT^2 \, (t - 23.5) \tag{4}$$

$$\Delta S^\circ \quad \frac{\Delta H^\circ - \Delta F^\circ}{} \tag{5}$$

These quantities are given in Table II.
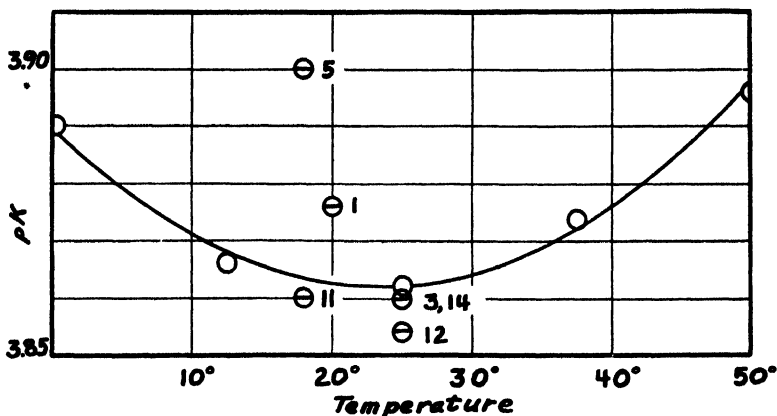
Fig. 3. The variation of pK of lactic acid with temperature. The smooth curve is a plot of Equation 2. ○ represents the present results. ⊖ represents results obtained by other investigators; the numbers refer to the bibliographic references.

SUMMARY

1. The values of the thermodynamic ionization constant, and the change in free energy, heat content, and entropy accompanying the ionization of lactic acid have been obtained for temperatures from 0–50° from electromotive force measurements of cells without liquid junction containing buffer solutions composed of hydrochloric acid and lithium, barium, strontium, calcium, or zinc lactate.

2. The ionization of lactic acid is greatest at 23.5°.

3. The value of pK at 25° is 3.862 and at 37.5° is 3.872.

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A NEW SYNTHESIS OF 1-AMINO-2-HYDROXYPROPANE
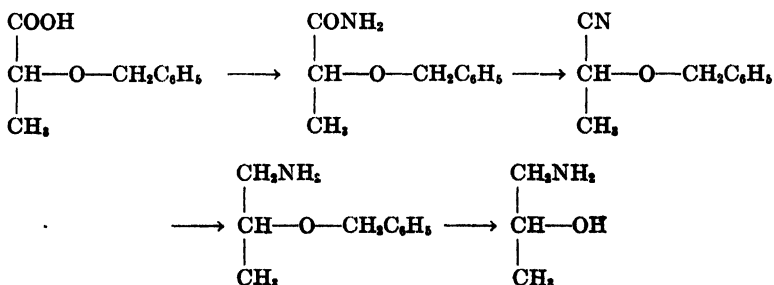
BY P. A. LEVENE

WITH THE ASSISTANCE OF MARTIN KUNA

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In this communication a method is described for the preparation of 1-amino-2-hydroxypropane which can be applied to the preparation of any one of the higher homologues. The advantage of the method lies in the fact that it permits the preparation of optically active substances. The starting material is benzoxypropionic acid which is converted into the nitrile by the usual set of reactions.

The nitrile can be reduced either to the stage of 1-amino-2-benzoxypropane only (by making use of Raney's catalyst) or directly into the free 1-amino-2-hydroxyamine by the use of colloidal palladium.

The principal reactions involved in the synthesis of the hydroxyamines are shown in the accompanying formulæ.

$$\begin{array}{ccccc}
COOH & & CONH_2 & & CN \\
| & & | & & | \\
CH-O-CH_2C_6H_5 & \longrightarrow & CH-O-CH_2C_6H_5 & \longrightarrow & CH-O-CH_2C_6H_5 \\
| & & | & & | \\
CH_3 & & CH_3 & & CH_3
\end{array}$$

$$\begin{array}{ccccc}
& & CH_2NH_2 & & CH_2NH_2 \\
& & | & & | \\
\cdot & \longrightarrow & CH-O-CH_2C_6H_5 & \longrightarrow & CH-OH \\
& & | & & | \\
& & CH_3 & & CH_3
\end{array}$$

The *dl*-benzoxypropionic acid is readily resolved into the active isomers and hence the active hydroxyamines are made readily accessible.

2-Benzoxypropionic Acid—14 gm.[1] of lithium metal were dissolved in 600 cc. of benzyl alcohol (distilled) and 155 gm. of α-bromopropionic acid (b.p. 92–95° at 12 mm.) were added dropwise with stirring. The reaction mixture was heated at 150° for 4 hours. It was then poured into water and extracted four times with ether, 500 cc. being used each time. The aqueous layer was acidified with sulfuric acid and extracted with ether. The extract was washed with water and dried over anhydrous sodium sulfate. B.p. 123–128° at 0.2 mm. Yield 145 gm.

0.1034 gm. substance: 5.678 cc. 0.1 N NaOH
        Mol. wt. Calculated, 180.1; found, 182.1
4.100 mg. substance: 9.975 mg. CO_2 and 2.528 mg. H_2O
        $C_{10}H_{12}O_3$. Calculated.   C 66.63, H 6.72
        180.1   Found.   " 66.34, " 6.90

Resolution of 2-Benzoxypropionic Acid—500 gm. of 2-benzoxypropionic acid were dissolved in about 4 liters of acetone and 750 gm. of cinchonidine added. The crystals were filtered and recrystallized from acetone twelve times. The acid obtained from a portion of the crystals was distilled. B.p. 125–128° at 0.5 mm.

$$[\alpha]_D^{25} = \frac{-6.60° \times 100}{1 \times 10.65} = -62.0° \text{ (in benzene)}$$

The precipitate was again recrystallized twice from acetone. The acid now had

$$[\alpha]_D^{25} = \frac{-6.15° \times 100}{1 \times 9.84} = -62.5°; [M]_D^{25} = -112.6° \text{ (in benzene)}$$

An acid which had a rotation of

$$[\alpha]_D^{25} = \frac{+3.85° \times 100}{1 \times 9.76} = +39.4° \text{ (in benzene)}$$

was obtained from the first mother liquors.

2-Benzoxypropionyl Chloride—80 gm. of *dl*-2-benzoxypropionic acid were refluxed with 140 cc. of freshly distilled thionyl chloride (b.p. 75–77°) during 30 minutes. The excess of thionyl chloride was distilled off at reduced pressure. The residue weighed 92 gm.

[1] Fischer, H. O. L., and Gohlke, B., *Helv. chim. acta,* **16,** 1132 (1933).

For analysis a small portion was distilled. B.p. 79–81° at 0.5 mm.

5.355 mg. substance: 11.900 mg. CO_2 and 2.810 mg. H_2O
$C_{10}H_{11}O_2Cl$. Calculated. C 60.44, H 5.58
195.6 Found. " 60.60, " 5.85

2-Benzoxypropionamide—92 gm. of 2-benzoxypropionyl chloride (not distilled) were gradually dropped into 400 cc. of a 25 per cent solution of ammonia in methyl alcohol at $-10°$. The amide was filtered off and recrystallized from ether and petroleum ether.

4.197 mg. substance: 10.250 mg. CO_2 and 2.755 mg. H_2O
$C_{10}H_{13}O_2N$. Calculated. C 66.99, H 7.31
179.1 Found. " 66.60, " 7.35

2-Benzoxypropionitrile—10 gm. of 2-benzoxypropionamide were dissolved in 30 gm. of thionyl chloride (b.p. 75–77°) and refluxed for 1 hour. The excess thionyl chloride was removed by distillation at reduced pressure. The substance was purified by distillation. B.p. 91–93° at 1 mm. Yield 8 gm.

3.880 mg. substance: 0.297 cc. N_2 at 24° and 757 mm.
$C_{10}H_{11}ON$ (161.1). Calculated, N 8.70; found, N 8.76

2-Benzoxypropylamine—1.5 gm. of 2-benzoxypropionitrile in methyl alcohol were reduced with hydrogen in the presence of Raney's catalyst in alcohol. The solution took up the required volume of hydrogen in 2 hours. The filtrate from the catalyst was acidulated with alcoholic hydrogen chloride and was concentrated to a small volume. The solution was filtered and platinum chloride solution was added to the filtrate. On addition of ether a precipitate settled out. This was filtered off and recrystallized from methyl alcohol, giving nice prismatic needles of a melting point of 207° (not corrected).

10.700 mg. substance: 0.366 cc. N_2 at 26° and 755 mm.
10.100 " " : 2.698 mg. Pt
6.537 " " : 7.810 " CO_2 and 2.420 mg. H_2O
$C_{20}H_{32}O_2N_2PtCl_6$. Calculated. C 32.42, H 4.36, N 3.80, Pt 26.37
740.3 Found. " 32.58, " 4.15, " 3.89, " 26.61

7 gm. of 2-benzoxypropionitrile were reduced as above. The hydrochloride was isolated by distilling the alcoholic hydrogen

chloride solution to dryness and evaporating with benzene. The hydrochloride was dissolved in a little absolute alcohol. The salt crystallized on addition of ether and pentane.

> 0.1000 gm. substance : 5.20 cc. 0.1 N HCl (Kjeldahl)
> $C_{10}H_{16}ONCl$ (201.6). Calculated, N 6.95; found, N 7.28

2-Hydroxypropylamine—1 gm. of 2-benzoxypropionitrile was added to 0.5 gm. of colloidal palladium in glacial acetic acid (saturated with 30 cc. of hydrogen) and shaken in an atmosphere of hydrogen. 488 cc. were taken up. The catalyst was removed by filtration, and the filtrate was acidulated with alcoholic hydrogen chloride and concentrated. The hydrochloride of the base was converted into the chloroplatinate. The precipitate was dissolved in hot methyl alcohol and filtered. When ether was added to the filtrate, the substance crystallized in small needles.

> 8.210 mg. substance: 0.372 cc. N_2 at 27.5° and 755 mm.
> 9.590 "   "   : 3.315 mg. Pt
> 9.590 "   "   : 4.550 " CO_2 and 3.075 mg. H_2O
> $C_6H_{20}O_2N_2PtCl_6$. Calculated.   C 12.85, H 3.60, N 5.02, Pt 34.85
> 560.2   Found.   " 12.94, " 3.59, " 5.11, " 34.57

GLUTAMINE AND ASPARAGINE IN TOBACCO LEAVES*

By HUBERT BRADFORD VICKERY and GEORGE W. PUCHER

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Attention has previously been drawn (1) to the presence in mature tobacco leaves of a substance that is decomposed by boiling water with the production of ammonia, and a recent study (2) of the composition of the tobacco plant at all stages of growth has shown that a similar substance is present both in the leaves and in the stalk throughout the life of the plant. The properties of this substance are those of glutamine, but the only way in which it can be made certain that glutamine actually occurs in a given tissue is to isolate the amide and identify it by chemical methods. Accordingly, a quantity of tobacco leaves has been subjected to a procedure designed to this end.

Previous observations have shown that enrichment of the amide content of tobacco leaves takes place during the first few days after removal from the plant (1); 13.4 kilos of mature leaves were therefore allowed to stand for 3 days, during which time the fresh weight diminished to 11.1 kilos, and the leaves became partly yellow. The tissue was then passed through a meat grinder and the pulp was pressed at the hydraulic press, whereby 9.8 liters of fluid were obtained. The press residue was shredded and suspended in ether for half an hour in order to cytolyze unbroken cells (3), and was again pressed out, another 0.6 liter of aqueous fluid being secured. The residue was then washed twice with water, being pressed each time. Analysis of the thoroughly mixed fluid gave the results in the second column of Table I. The procedure for the isolation of glutamine described by Vickery, Pucher, and Clark (4) was then followed.

The solution derived from the decomposition of the mercuric nitrate precipitate gave on analysis the results in the third column of Table I. The increase of ammonia nitrogen is doubtless due to the hydrolysis of a part of the glutamine during the manipulations. The loss of one-fifth of the asparagine is largely attributable to the removal of the voluminous basic lead acetate precipitate which was washed only once; it may be assumed that a similar proportion of the glutamine was also lost in this manner. Glutamine is quantitatively converted into ammonia and pyrrolidonecarboxylic acid by hydrolysis at pH 6.5; it is therefore possible to calculate the amino nitrogen of the amino acids by subtracting the amino nitrogen of the asparagine (equal to the

TABLE I

Composition of Extract from Tobacco Leaves and of Mercuric Nitrate Fraction Obtained from This during Preparation of Glutamine

	Extract	HgNO₃ ppt.	Loss
	gm.	*gm.*	*per cent*
Total N (including nitrate N) (5)...............	25.68		
Ammonia N (6).................................	0.525	0.565	
Glutamine amide N (7)........................	1.38	0.895	35.1
Asparagine " " (7)........................	2.26	1.80	20.4
Amino N (8)..................................	8.27	4.99	39.6
" " after hydrolysis at pH 6.5 (8)........	5.84	3.52	39.7
Loss of amino N after hydrolysis at pH 6.5......	2.43	1.47	39.5
Amino N of amino acids......................	3.58	1.72	52.0

amide nitrogen) from the amino nitrogen present after hydrolysis at pH 6.5. The disappearance of 52 per cent of the amino acid nitrogen indicates that the precipitation of the amides with mercuric nitrate effected a substantial simplification of the mixture. It should be noted, however, that this reagent is far from being a specific precipitant of the amides.

The mercuric nitrate fraction was neutralized with ammonium hydroxide and concentrated, as described by Vickery, Pucher, and Clark, to a sirup that was treated with alcohol and chilled. The crystalline material, which had separated overnight, was subjected to fractional recrystallization from aqueous and aqueous alcoholic mother liquors, whereby two successive crops of

asparagine of water of crystallization 12.13 and 12.05 per cent (theory 12.00), and two crops of glutamine of decomposition point 182.5° and 183° respectively were obtained. After being recrystallized once more these contained 18.5 and 19.1 per cent of nitrogen (theory for asparagine 18.67 per cent, for glutamine 19.18 per cent), and the glutamine decomposed at 185–186°.

The yield of asparagine amounted to 17.9 gm., which contain 1.67 gm. of amide nitrogen; this is 92.8 per cent of the quantity found by indirect analysis in the mercuric nitrate fraction, and 73.9 per cent of that in the aqueous extract from the leaves. The yield of glutamine was 6.04 gm., which contain 0.588 gm. of amide nitrogen; this is 65.7 per cent of the glutamine amide nitrogen in the mercuric nitrate fraction and 42.6 per cent of that in the original extract. Thus nearly three-quarters of the asparagine and somewhat less than one-half of the glutamine of the leaf extract were isolated in pure crystalline form. When the experimental difficulties of this operation are taken into consideration, it is clear that the results of indirect analysis of the extract from tobacco leaves may with some confidence be taken to represent a considerable part of the actual amide composition. The possibility that another amide or amide-like substance may be present is, however, not excluded, and observations already in hand (2) indicate that the stalk of the tobacco plant may contain such a substance. It is therefore necessary to emphasize that neither the results of isolation by the methods at present available, nor the results of indirect analysis, can be accepted as conclusive evidence that the entire amide composition of a plant tissue has been ascertained.

The tobacco plant furnishes an interesting example of an organism which synthesizes both glutamine and asparagine in response to certain stimuli, one of which at least appears to be ammonia (1). There is a marked contrast in this respect to the behavior of the tomato plant (9) and to that of the root of the common beet;[1] both respond to extraneous ammonia, that is, ammonia in the culture solution applied, with the almost exclusive production of glutamine. Many plants, on the other hand, appear to respond with the exclusive production of asparagine; it is necessary in this connection merely to refer to the work of Suzuki (10) and

[1] Unpublished observations.

of Prjanischnikow (11). Clearly, therefore, the further development of the theory of amide metabolism in plants will require not only a broadened knowledge of qualitative plant composition, but will necessitate at least a partial explanation of such facts as these.

SUMMARY

It has been shown that glutamine occurs, together with asparagine, in considerable quantities in the leaves of the tobacco plant. This furnishes an explanation of the production of ammonia when tobacco plant tissue is treated with boiling water. It has been pointed out, however, that this observation does not exclude the possibility that other amides, or amide-like substances, may also be present, and the importance to the understanding of the amide metabolism of plants of further qualitative study has been emphasized.

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THE COPPER CONTENT OF SOME HUMAN AND ANIMAL TISSUES

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The past few years have seen the study of copper metabolism step into the limelight along with that of iron and other metals which occur in small quantities in the body. This has been due partly to the work of Mallory (8) and others in following the high incidence of copper in the liver in cirrhosis and hemachromatosis, and partly to the relationship of the metal to the nutritional anemia as studied by Hart, Steenbock, Elvehjem, and their associates (3, 5, 13) and other groups of investigators.

More recently Hill (6) has found that the administration of copper results in the reduction in amount of inorganic iron stored in the liver, with a corresponding increase in the organic form. Josephs (7) has reported similar results.

Sachs (10) and his associates have reported a relationship between copper and iron levels in the blood of humans. When the iron content decreases, the copper content increases. This was shown to hold in a large series which included cases of pernicious anemia, sickle cell anemia, and leucemia. In pregnancy also they regularly find a hypercupremia (11) and call attention to the fact that anemia often accompanies pregnancy.

Sarata (12) finds hemorrhage is followed by hypercupremia, the increase being found chiefly in the red cells. He also finds that the cells which are poured into the blood stream in the emergency due to hemorrhage seem to contain more copper than usual.

We tabulate below the copper concentration found in human and animal tissues. It was planned to carry on the study of copper storage in animal tissues at the same time the iron storage was investigated (4). As this study cannot be continued in this

laboratory it seemed best to put on record our observations which may be of interest to the workers in this field.

Methods

The tissues from humans and normal dogs were used as obtained at autopsy or operation. All tissues from the anemic dogs were blood-free. The detailed protocols of these anemic animals, the methods of producing anemia, and the method of perfusion are to

TABLE I

Copper Content of Organs of Normal and Anemic Dogs

Dog No.	Condition	Copper and iron in fresh tissue					
		Liver		Spleen		Kidney	Lung
		Cu mg. per kg.	Fe mg. per cent	Cu mg. per kg.	Fe mg. per cent	Cu mg. per kg.	Cu mg. per kg.
V.E.-1	Normal	23		3.1		11	
V.E.-2	"	15		1.5		4.0	1.4
33-311	"			1.4			
33-292	"			1.5			
34-4	Eck fistula, intoxicated			4 3			
32-44	Anemic (400 mg. Fe orally)	26	1.4	26	8 6	14	
32-374	" (2000 " " ")	15	1.9	15	8.7		
33-1	" (216 " " intravenously)	59	26.8	25	75.2		
33-92	Anemic (108 mg. Fe intravenously)	61	18.1	13	49.8		
33-190	" (2800 " " orally)	50	5 0	7.0	11.2	12.3	2.8
27-235	" (2000 " " ")	20	17.8	5.1	10.4		
33-85	" (amino acids fed, fasting)	35	1.7	5.0	7 9		
33-325	" (liver and liver extract fed)	47	1 3	15	9.4		

be found in detail in another paper (4). These animals had been maintained at an anemic level of about ⅓ normal hemoglobin by means of blood removal for periods of at least 2 months preceding death.

Weighed samples of fresh tissues were placed in Kjeldahl flasks and 40 ml. of fuming nitric acid and 20 ml. of concentrated sulfuric acid were added. The material was brought to a boil and heating continued until charring became apparent. Perchloric acid (60 per cent) was then added, 2 ml. at a time, at intervals (from 2 to

10 ml. in all) until the solution was colorless or pale yellow turning to colorless on cooling. The cooled digestion products were then transferred to volumetric flasks by means of water distilled from glass. Aliquots were then analyzed for copper by the chromotropic acid method as described by Ansbacher, Remington, and Culp (1).

TABLE II

Copper Content of Some Human Organs

Age	Sex	Case No.	Diagnosis	Cu content, mg. per kilo fresh weight		
				Liver	Spleen	Others
5 mo. fetus	F.	X-5130	Prematurity	78		
Still-born	M.	X-5113	Undetermined	67		
"	"	X-5274	"	42		
3 days	"	A-2187	Hemorrhage	54		
1 wk.	"	A-2377	Hydrocephalus, meningitis	55		
9 wks.	"	A-2209	Pneumonia	34		
5 yrs.	"	A-2414	Mediterranean anemia	12		Lung 4.8, rib 19
7 "	"	X-5812	" "	15	2.3	
38 "	F.	A-2433	Cancer, cervix; cholecystitis	9.5		
68 "	"	A-2200	Cancer, ovary; acute hepatitis	11		
15 "	"	82172-S	Banti's disease, splenectomy		2.3	
68 "	"	A-2577	Myeloid leucemia	3.3	2.9	
78 "	"	A-2555	Melanoma of liver	4.2		Tumor nodule 4.5
60 "	M.	A-2729	Acute splenic tumor (bacteriemia)		1.0	

DISCUSSION

One striking observation is recorded in Table I; that, as the iron store in the spleen is depleted in anemia the copper store heaps up to high levels. To a less degree the same tendency is noted in the anemic dog liver. In the cases of anemic dogs not showing depleted iron stores in the liver and spleen, the increases in iron content are the result of iron administration preceding death. It is not felt

that these transient changes grossly affected the copper stores. Just what this means physiologically is a matter for speculation, but we have not enough data to permit elaboration of any hypothesis.

There is a dearth of information in the literature concerning the copper in the tissues of dogs. Rost and Weitzel (9) cite a value for liver of 24 mg. per kilo of fresh weight. Cunningham (2) reports three values for dog liver on the basis of dry weight with an average of 88 mg. per kilo, which would correspond to about 20 to 25 mg. per kilo of fresh weight. The values in Table I are of the same order and would indicate a somewhat higher value than found in the livers of humans.

The values for copper of infants' livers in Table II are in accord with the findings of other investigators, showing the high values usually associated with early life.

The amount of copper in the normal adult human liver, as reported in the literature by fourteen investigators, shows that the range is from 0.6 to 17.5 mg. of Cu per kilo of fresh weight in 142 cases reported, with an average value of 6.14. The values in Table II are well within these limits in spite of the pathology represented. It might be pointed out that two cases of Mediterranean anemia occur in the higher bracket of normals.

SUMMARY

In experimental anemia in dogs the copper stores in the spleen rise to very high levels as the iron content falls to the lowest levels. There is also the same tendency in the liver for the copper stores to increase as the iron is depleted in anemia.

Human disease in this small series does not appear to modify the copper stores in any conspicuous fashion, but the *normal* base-line is none too securely established.

In Mediterranean anemia the liver copper is observed to be a high normal in spite of the fact that these tissues are very rich in iron (hemosiderin)—often 10 times the normal iron concentration.

Fetal and infant livers show the high values reported in many other papers.

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THE RELATIONSHIP BETWEEN SPECIFIC GRAVITY AND PROTEIN CONTENT IN PLASMA, SERUM, AND TRANSUDATE FROM DOGS

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In 1930 Moore and Van Slyke (1) demonstrated that the relationship between the specific gravity and protein content of plasma was much closer than that between refractive index and protein content. The relationship was expressed by the equation of a straight line.

$$P = 343 \ (G - 1.0070)$$

in which P represents the gm. of total protein per 100 cc. of plasma and G its specific gravity. In a series of 118 observations the maximum deviation of the chemically determined protein content from that estimated by the specific gravity was 0.6 gm. per cent. Other investigators (2) have confirmed the accuracy of predictions based on this formula and in particular it has been shown (3) that variations in the albumin to globulin ratio alone do not affect the relationship to a measurable extent. Recently, however, Zozaya (4) has published data which are not in agreement with those of the aforementioned authors. He found very little correlation (correlation coefficient, 0.28) between the specific gravity and protein content of serum and a somewhat better correlation (correlation coefficient, 0.53) between gravity and refractive index. Zozaya thought that the failure of his findings to agree with those of Moore and Van Slyke might result from the use of serum instead of plasma.

During the past several years numerous samples of plasma, serum, lymph, ascitic fluid, and subcutaneous edema fluid taken from dogs have been analyzed in this laboratory both for specific

gravity and protein content.   Because of the variant nature of the different types of samples and because they were obtained from animals in which the development of hypoproteinemia was being studied, a wide range of values has been encountered.   Such measurements are particularly well adapted for determining the relationship between gravity and protein; in view of present disagreement a report on the findings appears to be justified.

Methods

The blood was obtained by puncture of the femoral artery and the plasma or serum separated by the usual process of centrifugation.   When plasma was desired, clotting was prevented by the addition of 1 mg. of heparin to each cc. of blood.   Lymph was secured by means of a cannula inserted in one of the large lymphatic trunks of the ankle.   Ascitic fluid and subcutaneous edema fluid were withdrawn after puncture of the peritoneum and edematous subcutaneous tissue, respectively, with a No. 18 gage needle.   Fibrin clots in these fluids were removed by centrifugation before analysis.   Nitrogen was determined in all fluids, both before and after the removal of protein with tungstic acid, by a micro-Kjeldahl procedure which has been described previously (2).   Protein was calculated as 6.25 times the difference between total nitrogen and non-protein nitrogen.   Specific gravity was determined in 2 cc. pycnometer bottles, as described by Moore and Van Slyke (1).   The measurements were made at room temperature and calculated for 20° by means of corrections given by these authors.   The same corrections were applied to all fluids, regardless of their protein content, since an examination of the data presented by Moore and Van Slyke indicates a negligible difference through ordinary ranges of room temperature between the expansion of plasma and pure water.

Standard methods of statistical analysis, based on the theory of least squares, were used in evaluating the relationships between specific gravity and protein content.[1]   Because the several coefficients of correlation were extremely high, in the neighborhood

[1] The formulæ for computing correlation coefficients, regression equations, errors of estimate, and errors of measurement are given in most reference works on statistics.   All formulæ used in this analysis are given by Garrett (5).

of 0.99, it was necessary to avoid short cuts in calculation such as are afforded by scatter diagrams, and to make all computations by the more laborious long method. The accuracy of these coefficients was maintained to six places of decimals.

Results

For purposes of correlation the data were arranged in three groups according to the nature of the samples: (1) plasma, (2) serum, and (3) edema fluid, ascitic fluid, and lymph. Both regression lines were computed; that is, one giving the best prediction of protein from specific gravity, and the other the best prediction of gravity from protein. It should be emphasized that the two regression lines are distinct and cannot be obtained one from the other by algebraic transposition. The following abbreviations are employed: r represents the coefficient of correlation, P refers to gm. of protein per 100 cc., and G is the specific gravity. The figure after the plus and minus sign is the statistical "probable error of estimate."

Relationship between Specific Gravity and Protein Content of Plasma—The results on 164 samples of plasma were available for this correlation. The range covered by the observations is shown by the following mean values with their standard deviations: for total protein, 4.96 gm. per cent, s.d. 1.17 gm. per cent, and for specific gravity, 1.0214, s.d. 0.0034. Statistical analysis yielded the following relationships.

$$r = 0.991532$$

(1) $$P = 340.1 \ (G - 1.00687) \pm 0.103$$
(2) $$G = 0.002890P + 1.00712 \pm 0.00030$$

Relationship between Specific Gravity and Protein Content of Serum—The results on 145 samples of serum were available for this correlation. The range is shown by the mean values and standard deviations: for total protein, 4.83 gm. per cent, s.d. 1.01 gm. per cent, and for specific gravity, 1.0211, s.d. 0.0029. Statistical analysis yielded the following relationships.

$$r = 0.993734$$

(3) $$P = 347.9 \ (G - 1.00726) \pm 0.076$$
(4) $$G = 0.002838P + 1.00743 \pm 0.00022$$

The correlation coefficient in this group of sera is slightly higher than in the preceding series of plasmas even though the range of the observations is somewhat less. This circumstance, which results in an appreciably lower value for the probable error of estimate in the formulæ for serum, probably finds its explanation in the fact that our first analytical efforts were with the plasmas and that the technical experience gained with them was later reflected in more consistent analyses when serum was later subjected to study.

Relationship between Specific Gravity and Protein Content of Transudate from Blood—This series embraced two samples of ascitic fluid, seven samples of edema fluid, and forty-seven samples of lymph,—a total of 56 fluids. The mean values and standard deviations are: for total protein, 0.86 gm. per cent, s.d. 0.53 gm. per cent, and for specific gravity, 1.0100, s.d. 0.0015. Statistical analysis yielded the following relationships.

$$r = 0.986418$$
(5) $$P = 353.1 \ (G - 1.00759) \pm 0.058$$
(6) $$G = 0.002755P + 1.00766 \pm 0.00016$$

In the analysis of transudates by the Kjeldahl method we have routinely used larger samples than with sera and the analytical error in determining protein has been less for transudates than for sera. It will be shown presently that when this fact is allowed for the errors of estimate in Equations 5 and 6 are not significantly different from those in Equations 3 and 4.

Relationship between Specific Gravity and Protein Content of Serum and Transudate Collectively—If from Equations 4 and 6 the most probable specific gravity of the ultrafiltrate from serum or from a transudate, respectively, is calculated by placing P equal to 0, the figure 1.00743 is obtained for serum ultrafiltrate and the figure 1.00766 for transudate ultrafiltrate. The fact suggests that in general biologic fluids of high protein content contain a slightly lower amount of non-protein solid material than do fluids of low protein content. This circumstance, which is in accord with theoretical expectation, in turn suggests that the relationship between gravity and protein in biologic fluids in general may be expressed more accurately by the equation of a line which is slightly curved rather than by one which is straight. Accordingly

the statistical test for linearity of regression was applied to the 201 observations on sera and transudates collectively. The result discloses that the apparent digression from linear relationship is too slight to have statistical significance. With this fact in mind the usual regression lines for the complete series of 201 observations were calculated with the object of obtaining equations which would fit satisfactorily the entire range of protein concentrations likely to be encountered in the several biologic fluids. The result of this correlation follows.

$$r = 0.998538$$

$$(7) \qquad P = 355.5\,(G - 1.00757) \pm 0.0727$$

$$(8) \qquad G = 0.002805P + 1.00760 \pm 0.00020$$

From the nature of the material and the preceding correlations it is apparent that the errors of estimate are greater in the high protein range than in the low. The figures for probable error of estimate in Equations 7 and 8 do not, therefore, possess an exact meaning for individual predictions but represent rather the average error in the series as a whole.

DISCUSSION

The correlations between specific gravity and protein content in this series of observations (Chart 1) are extremely high and the agreement between determined values for protein and those predicted from gravity by the several formulæ is even closer than in the series reported by Moore and Van Slyke (1). Abnormal values for blood lipid do not occur in the hypoproteinemic dog, while many of the plasmas studied by Moore and Van Slyke were obtained from patients with chronic nephritis and must have been abnormal in their lipid content. This circumstance undoubtedly accounts for the better correlation in the present series. In any case the present study confirms the findings of these earlier investigators, shows that there is no essential difference in correlation among data obtained from plasma, serum, and transudates, and in this respect fails either to agree with the findings of Zozaya (4) or to support his suggested explanation for them.

It may be inquired to what extent the several equations in this paper differ from each other and from the formula of Moore and Van Slyke. In Table I are presented values for total protein
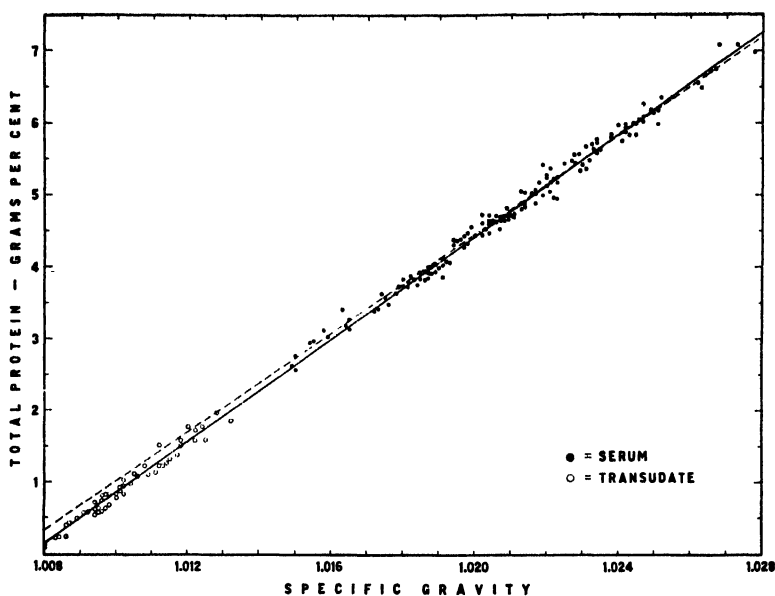
CHART 1. Relationship between specific gravity and protein content in the combined group of sera and transudates from dogs. The solid line is drawn from Equation 7 of this paper; the broken line gives the relationship as expressed by the formula of Moore and Van Slyke for human plasma.

TABLE I

Protein Concentration per 100 Cc. of Sample Predicted from Specific Gravity by Different Formulæ

Specific gravity	Source of prediction equation*				
	Human plasma	Dog plasma	Dog serum	Dog transudate	Dog serum and transudate
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1.0080	0.34	0.38	0.26	0.14	0.15
1.0100	1.03	1.06	0.95	0.85	0.86
1.0150	2.74	2.77	2.69	2.61	2.64
1.0200	4.46	4.47	4.43	4.38	4.42
1.0250	6.17	6.17	6.17	6.15	6.20
1.0300	7.89	7.87	7.91	7.91	7.97

* From left to right the formulæ for predicting the protein are: the equation of Moore and Van Slyke and Equations 1, 3, 5, and 7, respectively, given in this paper.

predicted by each of the equations from specific gravity readings throughout the range encountered in biologic fluids. It is apparent that all equations yield similar predictions throughout the range of protein concentration present in serum and plasma. However, those equations which are derived from plasma and serum observations alone yield high predictions for protein when applied to transudates. The close agreement between values for protein predicted by our formula (Equation 1) for dog plasma and those predicted by the formula of Moore and Van Slyke for human plasma suggests that the formulæ are interchangeable within practical limits between the two species.

Interpretation of Error of Estimate

The probable error of estimate in the preceding equations is a measure of the accuracy with which protein content can be predicted from specific gravity or *vice versa*. Its exact meaning can be clarified by example. In Equation 1 for plasma the probable error of estimate of protein from gravity is 0.103 gm. per cent. This figure means that if a large number of estimates of protein are made by use of the equation and compared with actual determined values of the protein, the difference between determined and estimated values will not exceed 0.103 gm. per cent in 50 per cent of the cases. Three variable factors are concerned in producing the error of estimate: (1) the error involved in protein analyses, (2) the error involved in determining gravity, and (3) variations in the non-protein constituents of biologic fluids, which do not affect the protein content but which do modify the gravity. It is of interest to determine how close a relationship between gravity and protein could be expected if all errors of measurement were eliminated. The knowledge can be had from the figures secured in duplicate determinations of gravity and protein and which allow computation of the errors of measurement. Equation 3 for serum tells us that the probable error of estimate of protein from gravity is 0.076 gm. per cent. In this series the probable error of measurement of protein was 0.023 gm. per cent and the protein equivalent of the probable error of measurement of gravity was 0.032 gm. per cent. The sum of the two errors of measurement is then 0.040 gm. per cent ($\sqrt{0.023^2 + 0.032^2}$) and the probable error of estimate of protein which would obtain if the errors of measure-

ment were removed is 0.065 gm. per cent ($\sqrt{0.076^2 - 0.040^2}$).
A similar calculation based on the data for transudates shows that
the probable error of estimate of protein in Equation 5 would be
reduced from 0.058 gm. per cent to 0.051 gm. per cent if the errors
of measurement were eliminated. As the corrected probable error
of estimate of protein for transudates, 0.051, does not differ
significantly from that for sera, 0.065, there is no proof that non-
protein solid constituents are not just as variable in transudates as
in sera. The fact that removal of errors of measurement results
in a slight decrease only in the errors of estimate means that at-
tempts to devise methods for measuring specific gravity more
accurately are not justified if the measurements are to be used for
prediction of protein.

SUMMARY

Measurements obtained from 164 samples of dog plasma, 145
samples of dog serum, and 56 samples of dog transudate have been
analyzed to determine the regression equations which express the
relationship between specific gravity and protein content. The
equation for dog plasma which permits the prediction of protein
from gravity is almost identical with that of Moore and Van Slyke
for human plasma. Likewise, the equations for dog plasma and
dog serum yield predictions which are almost the same throughout
the usual range of concentration. All equations for plasma and
serum yield slightly high predictions when applied to transudates.
It has been possible to calculate formulæ which give satisfactory
predictions throughout the entire concentration range encountered
in transudates and sera.

The ultimate accuracy with which protein could be predicted
from gravity, if all errors of measurement were eliminated, has
been computed. It is shown that removal of such errors would
improve only slightly the accuracy of prediction.

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THE AVAILABILITY OF *dl*-AMINO-N-METHYLHISTIDINE FOR GROWTH*

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In a recent investigation, Gordon and Jackson (1) have made the interesting observation that *dl*-amino-N-methyltryptophane is capable of stimulating growth in rats subsisting on a diet deficient in tryptophane. This finding was made even more striking by the demonstration that two other isomeric monomethyltryptophanes, namely Bz-3-methyltryptophane and Pr-2-methyltryptophane, are ineffective in this respect. These authors suggest that "other N-methylamino acids may be metabolized and utilized in place of the corresponding natural amino acids." It appeared to be of considerable interest to determine whether the results of Gordon and Jackson could be extended to N-methylamino derivatives of other essential amino acids. The present communication records experiments designed to test the availability of *dl*-amino-N-methylhistidine for the growth of rats subsisting on a diet deficient in histidine. An intermediate in the preparation of the amino-N-methylhistidine, namely *dl*-α-chloro-β-imidazolepropionic acid, has also been subjected to the method of animal assay employed in this investigation.

EXPERIMENTAL

The *dl*-amino-N-methylhistidine used in these experiments was synthesized according to Fargher and Pyman (2). *dl*-Histidine monohydrochloride was converted to α-chloro-β-imidazolepropi-

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onic acid by means of concentrated hydrochloric acid and sodium nitrite.   The product was recrystallized twice from dilute alcohol; it melted at 188° (corrected).

Analysis
$C_6H_7O_2N_2Cl$.   Calculated.   N 16.04,   Cl 20.34
Found.   " 15.8, 15.9, " 20.22

The chloroimidazolepropionic acid was transformed into the amino-N-methylhistidine by treatment with methylamine.   The product was purified through the picrate and the regenerated amino-N-methylhistidine isolated as the dihydrochloride.   The compound was recrystallized twice from dilute hydrochloric acid. It melted at 134–135° (corrected).   Fargher and Pyman reported a corrected melting point of 134°.   The air-dried salt contains 1 molecule of water of crystallization which is not lost on heating at 60° in a vacuum.

Analysis
$C_7H_{11}O_2N_3 \cdot 2HCl + H_2O$.   Calculated.   N 16.16   Cl 27.30
Found.   " 15.97, 16.02, " 27.02, 27.24

The amino-N-methylhistidine gave a negative ninhydrin test. A determination of amino nitrogen in the Van Slyke apparatus demonstrated that no nitrogen in this form was present in the compound.

The testing of the two compounds described above for their ability to promote growth in rats maintained on a histidine-deficient diet was conducted in a manner similar to that recently employed by Cox and Berg (3) in comparing the availability of *d*- and *l*-histidine for growth.   The histidine-deficient amino acid mixture was prepared from hydrolyzed casein by the procedure described by Vickery and Leavenworth (4) for precipitating the histidine.   The amino acid mixture was not completely histidine-free.

Male rats at weaning were confined singly and placed on a histidine-deficient diet composed of the amino acid mixture poor in histidine 14.5, tryptophane 0.2, cystine 0.3, sucrose 15, lard 19, cod liver oil 5, salt mixture (5) 4, agar 2, and starch 40 per cent. Throughout the investigation, each animal received a daily supplement of 100 mg. of dried yeast.[1]   Animals placed on this diet

[1] Product of the Northwestern Yeast Company.

either failed to grow or gained weight at a rate much below that observed for rats placed at weaning on an adequate diet. When it had been definitely established that the animals had reached a relatively constant body weight, the derivatives to be studied were incorporated in the deficient diet. The molecular weight of *dl*-histidine monohydrochloride monohydrate,[2] 209.5, in mg. was chosen as 1 equivalent. 3 equivalents (628.5 mg.) of this compound were included in 100 gm. of the basal diet. In a similar manner, 3 equivalents (523.5 mg.) of the chloroimidazolepropionic acid and 3 equivalents (780 mg.) of amino-N-methylhistidine dihydrochloride monohydrate were incorporated in 100 gm. of the basal diet in the tests designed to determine the growth-promoting capacities of these two compounds. Chart I presents in graphic form the results obtained in this investigation.

<div align="center">DISCUSSION</div>

It is evident from the growth curves presented in Chart I that under the experimental conditions employed α-chloro-β-imidazole-propionic acid is incapable of stimulating the growth of animals maintained on a histidine-deficient diet. This finding is not entirely unforeseen in view of the ample evidence for the remarkable specificity which the animal organism frequently exhibits in its metabolic demands. The testing of this compound was of interest in order to study the ability of the rat to conduct a reaction which is readily carried out in the laboratory; namely, the replacement of an α-halogen atom in an aliphatic fatty acid by an amino group. From the results obtained, it is apparent that either the reaction of this type does not occur *in vivo*, or perhaps that the experimental conditions imposed on the animal are not optimum for this organic chemical reaction.

In contrast to the negative results with α-chloro-β-imidazole-propionic acid are those obtained with the amino-N-methylhisti-dine dihydrochloride. It is evident from the growth curves that this methyl derivative of histidine can be utilized for purposes of growth by animals ingesting the histidine–deficient diet. The growth stimulus resulting from the incorporation of amino-N-methylhistidine in the basal diet is striking and definitely similar

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[2] Obtained from Hoffmann-LaRoche, Inc. Nitrogen content (Kjeldahl), 19.89 per cent. Calculated for $C_6H_9O_2N_3 \cdot HCl + H_2O$, 20.05 per cent.

in all of the animals used in testing this derivative. With two
animals, *dl*-histidine monohydrochloride supplements were given
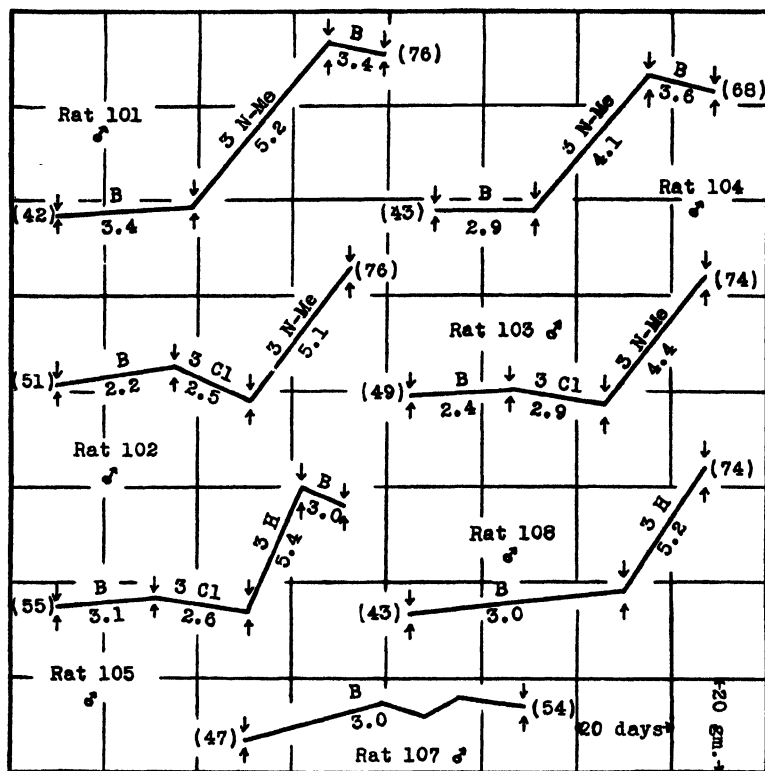

CHART I. Growth on basal histidine-deficient diet and on this diet sup-
plemented with amino-N-methylhistidine dihydrochloride, α-chloro-β-
imidazolepropionic acid, or histidine monohydrochloride. The diet em-
ployed during any part of an experiment is indicated by an abbreviation
between the two downward arrows representing the beginning and end of a
period. Diet B is a basal histidine-deficient diet; Diet 3 N-Me, Diet B
with 3 equivalents of amino-N-methylhistidine dihydrochloride mono-
hydrate; Diet 3 Cl, Diet B with 3 equivalents of α-chloro-β-imidazole-
propionic acid; Diet 3 H, Diet B with 3 equivalents of *dl*-histidine mono-
hydrochloride monohydrate. The average daily food consumption in gm.
for the corresponding interval is shown by figures inserted between the two
upward arrows. The initial and final body weights are presented in
parentheses.

in order to obtain some comparison between the growth-promoting ability of the amino acid and the synthetic methyl derivative. The average daily weight increment of the animals receiving the former compound was 1.6 gm.; the rats ingesting the diet supplemented with the amino-N-methylhistidine showed an average daily weight gain of 1.2 gm. Although the number of animals in each group is probably too small to permit drawing definite conclusions regarding the relative growth-promoting capacities of the two compounds, the uniformity of the results suggests that the slight difference observed may be a real one. Gordon and Jackson (1) reported that amino-N-methyltryptophane is not as effective a growth stimulant as *l*-tryptophane for rats subsisting on a tryptophane-deficient diet. These authors have adequately discussed the possible explanations of this type of result.

Grateful acknowledgment is made to Dr. W. G. Gordon and Dr. R. W. Jackson for making available to us prior to publication the results of their experiments (1) which suggested the present study.

SUMMARY

Amino-N-methylhistidine dihydrochloride has been found to be available for the growth of rats subsisting on a diet deficient in histidine. These experiments record the second successful replacement of an essential amino acid in the diet by its amino-N-methyl derivative.

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ON THE PRESENCE OF CREATININE IN BLOOD

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For a number of years, the literature has contained reports which have cast doubt on the validity of the concept that creatinine is present as such in normal blood.   In this paper, we wish to ‎present some data obtained by a study of the creatinine content, as measured by the alkaline picrate method of Folin (1), of ultrafiltrates of plasma and a comparison of these findings with those obtained on tungstic acid filtrates.   The use of an ultrafiltrate should eliminate any possible change in the nature of the chromogenic material in the original plasma, which might conceivably occur when protein precipitants are used.   Furthermore, an ultrafiltrate contains 5 to 10 times as much creatinine as do filtrates prepared by the use of a protein precipitant where a 1:5 or a 1:10 dilution of the blood is generally employed.   The alkaline picrate method is far more dependable when larger amounts of creatinine are present, and, therefore, the reactions of the creatinine in blood can be more readily studied by use of an ultrafiltrate.

The work of Hunter and Campbell (2) is probably the first to raise doubt as to the nature of the material in blood filtrates reacting with alkaline picrate.   From a study of rate of reaction curves, as determined by the rate of the color development in the Jaffe test in pure creatinine solution and in different types of blood filtrates, they reached the conclusion that in laked whole blood filtrates the results obtained were as much as 50 per cent higher than the actual creatinine content.   However, they state, "The Folin method determines the preformed creatinine of plasma with a satisfactory approximation to accuracy."   Essentially these conclusions were reached by Greenwald and McGuire (3) and by Wilson and Plass (4).   Work of a number of other authors indi-

cated that the preformed creatinine values obtained on laked blood filtrates were too high. In 1930, Folin and Svedberg (5) showed, by the use of the new unlaked blood filtrate (6), that the plasma contains more preformed creatinine than do the cells, due mainly to the difference in water content of the two. They state that the old higher values were due to the fact that disintegrated blood corpuscles give a substance or substances which react with the alkaline picrate reagent.

In 1922, Behre and Benedict (7) presented evidence which led them to conclude that preformed creatinine in blood does not exist in detectable quantities. In a recent paper (8) they reaffirm this view. Because the paper of Behre and Benedict has carried the most weight in favoring the view that creatinine is not present in normal blood in detectable quantities, and because their type of evidence and reasoning has been more or less the basis of later work, including this paper, it seems helpful to restate their evidence.

1. When sodium carbonate is added to picric acid, the depth of color in the solution increases slightly. In the presence of creatinine, in such quantities as are assumed in blood filtrates, this increase in color is slightly greater, but is scarcely more than detectable, and is not definitely proportional to the creatinine present. When carbonate is added to the picric acid filtrate from blood, there is a marked increase in color, and this color is far greater than could possibly be due to the creatinine content of the blood, as indicated by the regular determination.

2. Creatinine is destroyed by heating in alkaline solution. When tungstic acid filtrates from normal blood are heated with alkali, under similar conditions, the amount of color produced after treatment with alkaline picrate remains practically unchanged. However, when similar filtrates, with added creatinine, are treated in the same manner, the added creatinine is destroyed, while the original intensity of color was practically unaffected.

3. True creatinine is removed by kaolin quantitatively from pure solution or from tungstic acid-blood filtrates with creatinine added up to amounts corresponding to about 4 mg. of creatinine per 100 cc. of original blood. In heat coagulation filtrates, the removal of the creatinine-reacting substance of the blood by kaolin is irregular and uncertain, even when only small quantities

of the substance are present; *i.e.*, 30 to 50 per cent is removed. With picric acid filtrates (1:5 dilution) no appreciable amount of the chromogenic substance of normal blood is removed by kaolin, while added creatinine is removed satisfactorily.

Behre and Benedict, however, are not alone in having reached the conclusion that creatinine is not present in measurable amounts in normal blood. Gaebler and Keltch (9) and Gaebler (10) succeeded in isolating creatinine from normal blood as well as from retention blood. Nevertheless, the latter came to the conclusion that creatinine as such was not present in blood but was formed from some unknown precursor in blood by the action of the adsorbing agent (Lloyd's reagent) used in the method of isolation. Bohn and Hahn (11) also reached the conclusion that creatinine is not present in measurable amounts in normal blood in detectable quantities.

However, in the last few years a number of investigations have been recorded in which the experimental evidence has been interpreted as demonstrating that the major part of the chromogenic material in blood reacting with alkaline picrate is creatinine. Ferro-Luzzi (12) found that if pure solutions containing sugar or protein split-products were heated with sodium hydroxide, the products formed were capable of producing a color with alkaline picrate. By analogy he concludes that similar substances in blood filtrates produce chromogenic material when treated in the same manner, and that the creatinine originally present was destroyed. He finds that kaolin adsorbs the greater part of the chromogenic substance from a Somogyi zinc hydroxide blood filtrate. Also, he found that the chromogenic substance in blood reacts in identical manner with pure creatinine solution when treated with hot formaldehyde. The ability to develop a color with alkaline picrate is destroyed in both after such treatment.

Lieb and Zacherl (13), by use of the Pulfrich photometer and by measuring the extinction coefficient of the chromogenic material in blood reacting with alkaline picrate, came to the conclusion that the reaction of material in blood in the Jaffe reaction is completely analogous to creatinine and creatine.

Hayman, Johnston, and Bender (14), studying rate of color development in a manner similar to Hunter and Campbell, found that material eluted from Lloyd's reagent, which had been shaken

with a trichloroacetic acid-serum filtrate, gave curves identical with pure creatinine solutions. However, a small amount of color did develop in the serum filtrate after it had been treated with Lloyd's reagent. The rate of color development of this small fraction was far different from creatinine, but the amount of color was so small that its significance is open to question. Since this was a constant finding, Hayman, Johnston, and Bender interpret it as indicating that there is present in normal blood a small amount of material other than creatinine, which reacts with alkaline picrate.

Another interesting observation made by these workers is that the color produced on the addition of alkali to a pure picric acid solution previously treated with kaolin is a great deal more than that produced by adding alkali to an untreated picric acid solution. They conclude that the error caused by the above is sufficient to account for failure to detect the removal of any "creatinine" with kaolin from picric acid-blood filtrates.

<div align="center">EXPERIMENTAL</div>

Creatinine Determination—The method for the determination of creatinine is essentially that described by Folin and Wu (15). To 10 cc. of the ultrafiltrate or tungstic acid filtrate are added 5 cc. of a freshly prepared alkaline picrate solution, prepared by adding 5 cc. of 10 per cent sodium hydroxide to 25 cc. of a saturated solution of purified picric acid. The picric acid was purified by the very convenient and efficient method described by Folin (16, 17).

Source of Blood—All animal blood used in this work was obtained from normal animals. Two samples of blood were obtained from hospital patients upon whom a venesection was performed. Potassium oxalate was used as anticoagulant for all samples. To obtain the plasma the whole blood was centrifuged at room temperature.

Preparation of Filtrates—The preparation of the tungstic acid filtrates was based on the methods of Folin and Wu (15) and of Folin (6), with the exception that in most cases the final dilution was 1:5 instead of 1:10, as is normally used.

Plasma—Plasma-tungstic acid filtrates were prepared by adding to 1 volume of plasma 2 volumes of distilled water, 1 volume of 5

per cent sodium tungstate solution, and 1 volume of ½ N sulfuric acid; these were mixed well and then filtered.

Laked Blood—The laked blood filtrates were prepared by adding to 1 volume of whole blood 2 volumes of distilled water, 1 volume of 10 per cent sodium tungstate, and 1 volume of ⅔ N sulfuric acid, and filtering.

Unlaked Blood—The unlaked blood filtrates were prepared by adding to 1 volume of whole blood 3 volumes of a solution containing 1.5 per cent anhydrous sodium sulfate and 1.4 per cent sodium tungstate; they were allowed to stand 15 minutes with occasional gentle shaking and then with gentle shaking 1 volume of ⅓ N sulfuric acid was added. The mixture was then filtered through a good grade of fluted filter paper (18).

Plasma Ultrafiltrates—The ultrafiltrates were prepared by filtering undiluted plasma through pyroxylin membranes prepared according to Wilson and Holiday (19). The filtration was carried out in an apparatus adapted from that described by the latter workers. An efficient water pump was our source of suction.

The data recorded in this work were obtained from the filtrate passing through the membrane during the 1st hour of filtration. Pure creatinine solutions filtered through the same apparatus gave identical values with the original solution as determined by the alkaline picrate method. If any concentration of the filtrate did occur, the amount was negligible for the purpose of our experiments.

Comparison of Creatinine Content of Plasma, Ultrafiltrate, and Tungstic Acid Filtrate—In Table I are recorded creatinine values obtained on tungstic acid filtrates and ultrafiltrates prepared from the same samples of plasma. The agreement between the creatinine content of the two types of filtrates shows that all the creatinine is ultrafiltrable. In this respect, our results are in agreement with those of Achard, Lévy, and Potop (20), whose work was published while this work was in progress.

Adsorption of Creatinine from Filtrates by Kaolin and Its Elution—A series of pure creatinine solutions, containing 0.1 mg. of creatinine per 10 cc., was treated with kaolin according to the following procedure. To 10 cc. of the creatinine solution in a centrifuge tube were added 1 gm. of acid-washed kaolin and 1 cc. of 2 N oxalic acid. The tube was stoppered and placed in a shak-

ing machine for 5 to 10 minutes, centrifuged, and the supernatant fluid drained off.   The residue was washed by shaking with 5 cc. of water plus 0.5 cc. of 2 N oxalic acid, centrifuged, and the wash water discarded.   The creatinine was eluted from the kaolin by

TABLE I

Preformed Creatinine Values of Plasma Obtained from Ultrafiltrates and Tungstic Acid Filtrates

Blood sample No.	Type of blood	Plasma ultrafiltrate	Plasma-tungstic acid filtrate	Remarks
		mg. per cent	*mg. per cent*	
1	Beef*	1.49	1.29	Normal
2	Human*	1.12	1.23	Chronic myocarditis
3	"	2.66	2.72	Acute glomerular nephritis
6	Beef	1.94	1.96	Normal
7	"	1.76	1.4	"
8	"	1.67	1.58	"
9	"	1.80	1.75	"
10	"	1.78	1.67	"

* Tungstic acid filtrate diluted 1:10.

TABLE II

Creatinine Recovered by Kaolin from 10 Cc. of Pure Solution Containing 0.1 Mg. of Creatinine

Creatinine eluted from kaolin	Amount recovered
mg.	*per cent*
0.091	91
0.078	78
0.086	86
0.094	94
0.088	88
0.086	86
Average..........................	87

adding 6 cc. of water and about 0.5 gm. of magnesium oxide, mixing well, and shaking in the shaking machine for 5 minutes.   The kaolin was separated by centrifuging and the liquid was decanted into a tube graduated at the 25 cc. mark.   The residue was washed in the same manner with an additional 6 cc. of water.   The wash

TABLE III

Removal of Creatinine from Whole Blood and Plasma Filtrates by Kaolin

All values are expressed as creatinine in mg. per cent except where indicated.

Blood sample No.	Plasma ultrafiltrate				Plasma-tungstic acid filtrate				Laked blood filtrate				Unlaked blood filtrate			
	Before kaolin treatment	Recovered from kaolin	Recovery, per cent	After kaolin treatment	Before kaolin treatment	Recovered from kaolin	Recovery, per cent	After kaolin treatment	Before kaolin treatment	Recovered from kaolin	Recovery, per cent	After kaolin treatment	Before kaolin treatment	Recovered from kaolin	Recovery, per cent	After kaolin treatment
1	1.49	1.28	86													
2	1.12	0.96	86													
3	2.66	2.18	87													
4					0.81	0.76	93									
5	1.59	1.15	72		1.23	1.16	94									
6	1.94	1.45	75	0.23	1.96	1.37	70	0.56	1.95	1.33	68	0.49	1.63	1.12	69	0.46
7									1.56	1.08	69	0.46	1.36	1.03	76	0.44
8	1.67	1.3	78	0.14	1.58	1.11	70	0.48	1.76	1.24	71	0.49	1.38	1.17	85	0.43
9	1.80	1.29	72	0.16	1.75	1.36	78	0.46	1.65	1.38	83		1.57	1.27	81	
10					1.67	1.36	84									
Average........			79.4				81				73				78	

Blood Sample 4 was normal dog blood; No. 5, normal pig blood. All other samples were the same as the corresponding numbers in Table I.

water was added to the graduated tube. To the entire extract were added 5 cc. of fresh alkaline picrate solution; the mixture was diluted to a volume of 25 cc. with water, and, after 10 minutes, compared with an appropriate creatinine standard prepared at the same time. Before making the color comparison it was necessary to centrifuge the unknown sample, for a precipitate of magnesium hydroxide was always formed. The results from a series of six experiments with pure creatinine solutions treated in the above manner are recorded in Table II. The recovery of creatinine ranged between 78 and 94 per cent and averaged 87 per cent.

Similar experiments were performed on a series of simultaneously prepared plasma ultrafiltrates, plasma-tungstic acid filtrates, laked blood filtrates, and unlaked blood filtrates. The kaolin residue and the filtrate from these experiments were studied separately.

Kaolin Residue. The creatinine adsorbed by the kaolin was eluted in the same manner as when pure creatinine solutions were used. The amount of creatinine recovered ranged from 68 to 94 per cent for all filtrates studied. The amounts of creatinine recovered from each type of filtrate are in substantial agreement as is shown in Table III (third column of each section). The recoveries of creatinine from the blood filtrates (both in range and average) are of the same order as those obtained from pure solutions of creatinine. Such variations as did occur are not greater than the uncertainties involved in the procedures.

Supernatant Fluid—The supernatant fluids after the treatment with kaolin were neutralized with weak sodium hydroxide and 5 cc. of alkaline picrate added. The slight colors developed in this filtrate were read against creatinine standards. In the last column of each section of Table III are recorded these quantities, determined and expressed as creatinine. The values for all tungstic acid filtrates are larger than those of the ultrafiltrates. We do not believe that these values actually represent creatinine or any other chromogenic material in the original blood filtrate, but are due in large part to material added in the procedure. These interfering factors would reasonably be expected to be relatively more pronounced in the more dilute filtrates (tungstic acid 1:5 and sometimes 1:10) than in a more concentrated solution like the ultrafiltrate which contains from 5 to 10 times as much creatinine.

The average amount of color developed in the kaolin filtrate prepared from tungstic acid filtrates is about 30 per cent of that developed in the original filtrate, while in the case of the ultrafiltrate it is only about 10 per cent of the original color developed. We have not studied any bloods showing a high creatinine content and cannot, therefore, say how much chromogenic material would remain in such filtrates after the above treatment.

From these results it appears that the major part of the chromogenic substance in tungstic acid filtrates and ultrafiltrates is adsorbed by kaolin.

Action of Hot Sodium Hydroxide on Pure Creatinine Solutions and on Blood Filtrates—To 10 cc. of ultrafiltrate or tungstic acid filtrate in a tube graduated at 25 cc. were added 2.5 cc. of 10 per cent sodium hydroxide. The tube was placed in a boiling water bath for 1 hour. It was then cooled and carefully neutralized with a predetermined volume of approximately 2.5 N HCl solution. 5 cc. of fresh alkaline picrate solution were then added, diluted to 25 cc., and after 10 minutes the color comparison was made with an appropriate standard prepared at the same time.

Filtrates of the different types studied were treated with hot sodium hydroxide according to the procedure just described. The creatinine content before and the apparent creatinine content after treatment with sodium hydroxide are recorded in the second and third columns, respectively, of Table IV. Our values after treatment with hot sodium hydroxide are always lower than those obtained before such treatment. In most cases, however, the difference is not significant. Although the values obtained by Behre and Benedict from this type of experiment were more nearly equivalent, we feel that our results in the main confirm their findings.

There are several features which are far more pronounced in the ultrafiltrate after this treatment than in any of the tungstic filtrates. A reddish brown precipitate always develops in the ultrafiltrate when it is heated with sodium hydroxide. If this precipitate is filtered off, the filtrate still has a decided brown tinge. The same is sometimes true with tungstic acid filtrates, but to a less marked degree. The color produced in an ultrafiltrate heated with sodium hydroxide and treated with alkaline picrate has always a very different shade from that of the creatinine standard

used for comparison, even though the color of the original filtrate always matched perfectly. At times the shades were so different that no colorimeter reading was attempted. This is also true of the tungstic acid filtrates but to a less degree. Therefore, the values recorded in Table IV (second column of each section) can be considered only as approximate.

Action of Hot Sodium Hydroxide on Kaolin-Treated Filtrates— As shown above, blood filtrates that have been treated with kaolin contain only very small amounts of chromogenic material capable

TABLE IV

Results Obtained by Heating Blood Filtrates for 1 Hour with 2 Per Cent Sodium Hydroxide before and after Treatment with Kaolin

All values are expressed as creatinine in mg. per cent.

Blood sample No.	Plasma ultrafiltrate			Plasma-tungstic acid filtrate			Laked blood filtrate			Unlaked blood filtrate		
	Before heating with NaOH	After heating with NaOH	After treatment with kaolin and heating with NaOH	Before heating with NaOH	After heating with NaOH	After treatment with kaolin and heating with NaOH	Before heating with NaOH	After heating with NaOH	After treatment with kaolin and heating with NaOH	Before heating with NaOH	After heating with NaOH	After treatment with kaolin and heating with NaOH
1	1.49	1.0										
2	1.12	0.87										
4				0.81	0.65	0.5						
5	1.59	1.39	1.16	1.23	0.84	0.72						
6	1.94	1.6	1.74	1.96	1.26	1.15	1.95	1.6		1.63	1.07	0.93
7	1.76	0.85		1.4	0.54		1.45	0.7		1.28	0.67	
9	1.80	1.6	1.67	1.75	1.32	0.84	1.76	1.5		1.38	1.07	

of reacting with alkaline picrate (Table III, fourth column of each section). A series of kaolin-treated filtrates, containing 2 per cent sodium hydroxide, was heated for 1 hour in a boiling water bath. After the filtrates containing the sodium hydroxide were cooled and neutralized with hydrochloric acid, each filtrate was treated with 5 cc. of alkaline picrate solution. After the material stood for 10 minutes, the color developed was read against an appropriate standard prepared at the same time. In Table IV (third column of each section) are recorded the values obtained

in these experiments. It will be noticed that the amount of color developed in a kaolin-treated filtrate heated with sodium hydroxide is very nearly the same as that obtained by heating an untreated blood filtrate with sodium hydroxide.

The same results were obtained when Lloyd's reagent was used as the adsorbing medium instead of kaolin for the removal of the preformed creatinine. It is, therefore, evident that the color developed with alkaline picrate after boiling with sodium hydroxide is not related to the chromogenic material present in the untreated filtrate, but represents some other chromogenic material formed by the action of hot sodium hydroxide. We have made no attempt to establish what these substances are. Ferro-Luzzi (12) believes that they are glucose and protein split-products.

. *Effect of Hot Sodium Hydroxide on Material Eluted from Kaolin* —As shown in Table III, about 80 per cent of the chromogenic material in the blood filtrates studied can be adsorbed by and eluted from kaolin. In a number of experiments the material eluted from kaolin which had been shaken with the various filtrates was heated with 2 per cent sodium hydroxide for 1 hour. In every case the property of developing a color with alkaline picrate was destroyed after such treatment. In this respect the material adsorbed from the blood filtrates by kaolin reacts in an entirely analogous manner to pure creatinine solutions.

Sodium Carbonate Used As Alkali for Creatinine Determination— Since Behre and Benedict (7) found that a picric acid filtrate treated with sodium carbonate as the alkali for the determination of creatinine gave far more color than would be expected when compared with known creatinine solutions treated in the same manner, it was thought advisable to repeat this experiment on ultrafiltrates of plasma. This we have done, using the conditions described by Behre and Benedict; *e.g.*, 0.5 cc. of 20 per cent sodium carbonate per 10 cc. of picric acid was used. In one typical experiment, a sample of ultrafiltrate containing 0.196 mg. of creatinine per 10 cc. was compared with 10 cc. of a standard solution containing 0.2 mg. of creatinine. To each were added 5 cc. of a freshly prepared carbonate-picrate solution (20 cc. of saturated picric acid solution plus 1 cc. of 20 per cent sodium carbonate solution). After standing 10 minutes or even a half hour, the amount of color in each was practically identical. This

experiment was repeated, but with the addition of 1 cc. extra of 20 per cent sodium carbonate to each tube. The results were the same as when the smaller amount of carbonate was used. In no case have we found any greater increase in color in ultra-filtrates than would be expected from their creatinine content as determined by the regular procedure. After the above solutions had stood for 24 hours, there was a marked color development in the ultrafiltrate tube. This phenomenon is regularly encountered in the regular creatinine determination on ultrafiltrates. After standing for 24 hours, these solutions develop far more color than can possibly be accounted for by the glucose present in the amount of filtrate used. This effect is also noted in a tungstic acid filtrate, but to a much less degree. If creatinine is first removed from an ultrafiltrate by kaolin and then alkaline picrate added and allowed to stand 24 hours, this development of color still occurs. Undoubtedly the development of this color must start as soon as the alkaline picrate is added to the filtrate, but its rate is so slow that in 10 minutes it could hardly have an appreciable effect.

Adsorption by Norit—10 cc. of plasma ultrafiltrate were shaken for 5 minutes in a shaking machine with about 0.5 gm. of acid-washed norit plus 1 cc. of 2 N oxalic acid solution. It was then centrifuged. The supernatant liquid was decanted and after the oxalic acid present was neutralized, it was treated with 5 cc. of alkaline picrate solution. Hardly any more color was developed than was produced by the alkaline picrate itself. Pure creatinine solutions containing 0.1 and 0.2 mg. of creatinine were treated in the same manner and the results were the same. No chromogenic material could be eluted from the norit which had been shaken with the ultrafiltrates or with the pure creatinine solutions by making them alkaline with magnesium oxide or sodium carbonate. The creatinine of blood and of pure solution reacted in the same manner on treatment with norit.

<center>DISCUSSION</center>

Our experiments show that the major portion of the chromogenic material in the filtrates studied was adsorbed by kaolin. In tungstic acid filtrates the adsorption amounted to 70 to 94 per cent, and in ultrafiltrates it was from 72 to 86 per cent. In these

cases the creatinine adsorption was measured by determining the amount of creatinine eluted from the kaolin used. In three samples of ultrafiltrate, the adsorption of creatinine by kaolin was from 88 to 91.4 per cent, as determined by subtracting the amount of chromogenic material remaining after kaolin treatment from that originally present in the filtrate. In this connection it should be recalled that Greenwald and McGuire (3) reported that kaolin removed creatinine from heat-coagulated blood filtrates almost quantitatively; Behre and Benedict were able to remove from 30 to 56 per cent of the creatinine from the same type of filtrate; Ferro-Luzzi (12) adsorbed from 67 to 100 per cent of the creatinine from Somogyi zinc hydroxide-blood filtrates. There is, therefore, evidence that a large amount of the chromogenic material in tungstic acid filtrates, heat coagulation filtrates, zinc hydroxide filtrates, and ultrafiltrates can be adsorbed on kaolin. When picric acid filtrates were used by Behre and Benedict, they could show no loss of chromogenic material. Gaebler (10) confirms this fact. The conclusion seems unavoidable that picric acid in the presence of blood filtrate constituents hinders the adsorption of creatinine or during the process of protein precipitation a new chromogenic material is formed which is not adsorbed by kaolin. The work of Hayman, Johnston, and Bender (14) indicates that picric acid after having been shaken with kaolin causes a color production itself, which, according to these authors, can explain these results. Water or dilute oxalic acid that has been shaken with kaolin does not alter the amount of color produced by alkaline picrate when they are added to known amounts of creatinine or to blanks. Therefore, this effect does not enter into the type of experiments we have performed.

The fact that a blood filtrate, heated with alkali, can still produce approximately the same amount of color with alkaline picrate as originally, is not evidence against the presence of creatinine in blood, as our experiments clearly show. An ultrafiltrate which has been shaken with kaolin produces very little color when alkaline picrate is added. However, if such a kaolin-treated filtrate is heated for 1 hour with 2 per cent sodium hydroxide, it will produce about the same amount of color with alkaline picrate as a filtrate not treated with kaolin, but heated with alkali and alkaline picrate added. Furthermore, the material adsorbed by and

eluted from the kaolin reacts with alkaline picrate, but if this fraction is heated with 2 per cent sodium hydroxide, its chromogenic property is entirely destroyed. Our interpretation of these experiments is that the creatinine originally present in the blood is adsorbed by kaolin. On heating a kaolin-treated filtrate with sodium hydroxide, one or more new chromogenic substances are formed. This explains why Behre and Benedict found that added creatinine was destroyed by the hot alkali treatment but that the chromogenic material originally present was not destroyed. In fact, the material originally present was destroyed and new chromogenic material was formed in about the same proportion by the action of hot alkali on other blood filtrate constituents.

Behre and Benedict found a very pronounced development of color in picric acid-blood filtrates when sodium carbonate was added as the alkali in the creatinine determination. In similar experiments, using ultrafiltrates and tungstic acid filtrates, we could not find any such color development during the time usually allowed in a creatinine determination. It is tempting to suggest again that some change had occurred while precipitating the proteins from blood with picric acid, which resulted in the production of a chromogenic substance capable of producing a color with the picrate-carbonate solution.

In our experiments we have found no difference in the way blood creatinine and pure creatinine react. Both are removed to about the same extent by kaolin. The material liberated from the kaolin is destroyed by hot alkali. The amount of color developed when sodium carbonate is used as the alkali for creatinine determination is of the same order in pure solution and in ultra-filtrates.

SUMMARY

1. Kaolin adsorbs creatinine from plasma ultrafiltrates, tungstic acid filtrates, and pure solutions in about equivalent quantities; i.e., an average of about 80 per cent of the amount originally present.

2. Plasma ultrafiltrates and tungstic acid filtrates retain the property of producing a color with alkaline picrate after boiling with sodium hydroxide. If the creatinine is first removed by adsorption on kaolin, and then the filtrate boiled with sodium

hydroxide, the amount of color developed with alkaline picrate is about equal to that produced when the kaolin treatment is omitted. On the other hand, the material eluted from kaolin is destroyed by heating with sodium hydroxide.

3. If sodium carbonate is used as the alkali in the creatinine determination, the amount of color developed in an ultrafiltrate is about that which is expected from the treatment of an equivalent amount of pure creatinine solution in the same manner.

4. Our conclusion is that the greater part if not all the chromogenic material in normal plasma is creatinine.

I wish to express my gratitude to the late Professor Otto Folin for suggesting the work on this problem and his sympathetic advice during the early stages of its development.

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FAT METABOLISM IN PLANTS WITH SPECIAL REFERENCE TO STEROLS

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The almost universal occurrence of sterols in all parts of plants examined would suggest that these substances have a definite function in the metabolism of the plant. As yet the nature of this function is quite obscure. The solution of this problem has been attempted with little success by numerous investigators, usually through a determination of the quantitative changes in the sterols during germination of seeds in the light in contrast to the dark.

The physiology of the sterols including vitamin D has been well reviewed by Bills (1935). He pointed out that the formation of sterols in higher plants as well as in the lower can apparently take place at any stage of development. This ability to synthesize sterols appears early in young plants as shown by Schulze and Barbieri (1882), Schulze (1890), and Terroine, Bonnet, Kopp, and Vechot (1927).

Concerning the synthesis of sterols during the germination of seeds in the light in contrast to the dark a wide diversity of results has been obtained. Schulze and Barbieri (1882) found in the case of the lupin that the sterol content of the whole seedling after germination in the dark is greater, but, after germination in the light is less, than that of the ungerminated seed. Ellis (1918), working with wheat, found the opposite to be the case. The increase in sterol was much more marked during germination in the light than in the dark. Beumer (1933), on the other hand, obtained results with peas and beans which differed from any of those reported earlier. He found a marked synthesis of sterol during germination both in the light and in the dark, although it was somewhat greater during etiolated growth.

In view of the diversity of results reported by different investigators concerning the occurrence and synthesis of sterols in plants under different conditions of germination, it is not surprising that the question of their function in plants is still quite obscure. However, despite the lack of agreement in earlier work, it is now well recognized that during germination of seeds, while the fat present shows a rapid diminution, under certain conditions there is a marked synthesis of sterol. Moreover, the sterol may be present both in the free form and combined with fatty acids as esters.

So far as the available literature indicates, no investigations have been made to determine what relations exist between the free and esterified sterol in plant tissues during germination. Information concerning the changes in these relative to those in the total fat of seeds during germination might throw considerable light on the possible significance of the sterols in plants.

It was with this in mind that the problem was undertaken to determine the changes in the total and relative amounts of free and ester sterol in seedlings, germinated in the light and in the dark, as compared to the ungerminated seeds; and to correlate these changes with those occurring simultaneously in the other fatty constituents.

<div align="center">EXPERIMENTAL</div>

Sound soy beans (mammoth yellow and black Wilson) were germinated in various ways to determine the best means of procuring successful germination without mold. The following method proved to be the most satisfactory: The selected beans were treated for 5 minutes with a 1:1000 mercuric chloride solution to destroy mold spores, then rinsed with distilled water, and placed upon moist quartz sand in a dish of suitable size covered by an inverted glass funnel. The etiolated seedlings were germinated in a dark room maintained at the same temperature as that at which the normal plants were grown. In certain experiments, a nutrient solution, viz. Crone's solution, was used instead of distilled water. This solution had the following composition: water 2 liters, KNO_3 1.0 gm., $FePO_4$ 0.5 gm., $CaSO_4$ 0.25 gm., and $MgSO_4$ 0.25 gm.

The seedlings were removed from the germinators at 1, 2, and 3 week intervals and the moist weight determined. They were then

dried in a vacuum oven at 70–80°, constant dry weight being obtained in 24 hours. It was experimentally demonstrated that the iodine numbers of the fatty acids following this method of drying were as high or higher than those obtained following dehydration of the material in alcohol, the average values being 134 and 132, respectively. This indicated the absence of any serious oxidative changes during drying.

A known amount of the dry plant material (5 to 15 gm.) was prepared for extraction by grinding, in a mortar with the aid of clean sand, into a fine powder. The ground material was quantitatively transferred into a paper extraction thimble which was suspended in a 500 cc. Erlenmeyer flask fitted with a block tin condenser. Continuous extraction was carried out with 75 cc. portions of boiling alcohol and ether for a total of 25 hours, the extract being removed and the solvent renewed at intervals. The first extraction, which removed most of the fatty material, was carried out for only 4 hours with alcohol alone. This was followed by three successive 7 hour periods of extraction, with 2:1 alcohol and ether the second and third periods and ether alone the last period. This procedure was found to remove all the fat, yet reduced the possibility of changes in the fatty material present due to the prolonged heating. The combined extracts were cooled, filtered, made up to a given volume, and preserved in a dark, cool place for analysis.

Since there are obvious advantages in using a procedure made up of several methods each based on the same principle, the micromethods of Bloor (1928), based on the oxidative principle, were used throughout the investigation. Thus, if any errors were involved, they were the same throughout. Moreover, having the determined lipid values in terms of the same unit facilitated calculation of values for lipids not directly determined. By the use of these methods it was possible to determine, directly, the total fatty acids, the iodine number of total fatty acids, the total sterol, and the free sterol. The value for the ester sterol was obtained by subtracting the value for free sterol from that for total sterol.

Total fatty acids were determined by the oxidative method of Bloor (1928). Preliminary investigation on known alcohol-ether solutions of fatty acids gave a recovery of 98 to 99 per cent by this

method.   The iodine numbers of the fatty acids were determined
by the method of Yasuda (1931–32).   These were carried out on
duplicate samples such as were used for the oxidative determina-
tion of the total fatty acids, which gave directly the amount of
fatty acids used.

It had been shown by Windaus (1909, 1910) that sterols are
quantitatively precipitated by digitonin as the insoluble digi-
tonide.   Moreover, this method had been found to be satisfactory
by Ellis (1918) and Beumer (1933) for the quantitative deter-
mination of plant sterols.   Consequently the procedure of Okey
(1930), as modified by Yasuda (1931), for the determination of
cholesterol by precipitation and subsequent oxidation as the
digitonide was adopted.

Preliminary experiments with this method showed that it offered
some difficulty in the way of reproducibility and accuracy of
results.   However, modifications were introduced by which a
satisfactory method was finally developed; the details of this
method have been described by Boyd (1933).   The results ob-
tained on known solutions of phytosterol, by use of this method,
although possibly 10 per cent low, were very consistent.

Results

The results of the analyses of ungerminated soy beans and beans
germinated for 1, 2, and 3 week periods are presented in Fig. 1.
The values, expressed in terms of dry weight, are the averages of
several analyses of lots of 100 to 125 seeds or seedlings.   How-
ever, it is a common observation that the total dry weight of
germinating seeds decreases as germination proceeds, the decrease
usually being slightly greater during germination in the light.
Thus the values for the fatty substances calculated on the basis of
dry weight would show an apparent increase after germination
even if the amount of fatty material remained constant.   For this
reason the percentage changes in the sterol during germination
have also been calculated in terms of the amount originally pres-
ent in the ungerminated beans.   These values will be used in
discussing the observed changes.

The ungerminated seeds of the two varieties of soy bean were
quite similar in their fat composition.   The total fatty acids
amounted to 17 to 18 per cent of the dry weight.   The sterol on

the other hand amounted to only 90 to 97 mg. per cent of the dry weight and was 85 to 90 per cent free sterol.

The rate of growth not being very uniform the seedlings germinated in water for 1 week were divided, for analysis, into groups according to size. During this period the percentage of fat showed a decrease from about 17 per cent to 11 to 12 per cent. At the same time there was a gradual synthesis of sterol, the largest seedlings showing an increase of 26 and 28 per cent after ger-
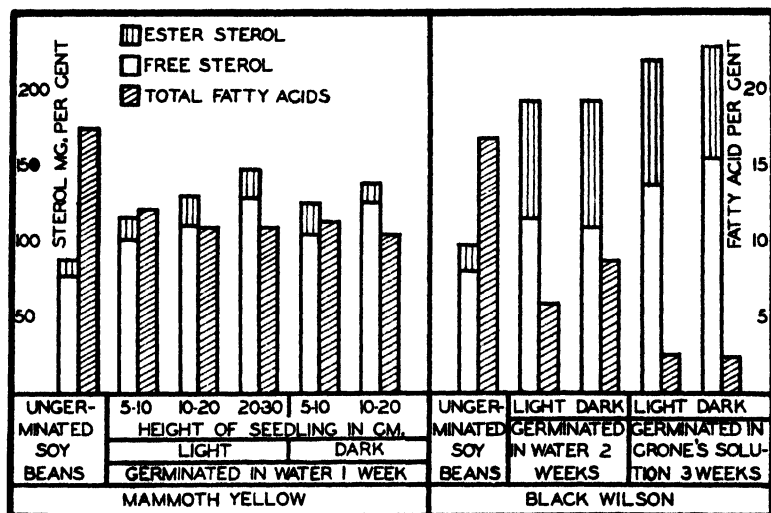

Fig. 1. Showing the total fatty acids in per cent and the total, free and ester sterol in mg. per cent of dry weight of mammoth yellow and black Wilson soy beans, ungerminated and germinated in the light and in the dark for 1, 2, and 3 week periods.

mination for 1 week in the light and the dark, respectively. The increase was limited chiefly to the free sterol, the ester constituting only 12 to 13 per cent of the total.

After 2 weeks of germination there was a further decrease in the percentage of fat. This was more marked during germination in the light than in the dark, the average values for the total fatty acids being 5.7 and 8.6 per cent, respectively. On the other hand, there was a continued increase in the sterol content amounting to 67.5 and 70 per cent in the light and dark, respectively. However,

the increase in sterol during this period was confined chiefly to the ester fraction such that it constituted 41 and 44 per cent of the total sterol for the light and dark, respectively.

Invariably the plants showed definite signs of wilting by the end of 2 weeks germination, no doubt due to the depletion of essential constituents in the stored reserves. Consequently, for later periods of growth it was necessary to use a nutrient solution. By the use of Crone's solution germination could be continued satisfactorily for 3 weeks. Fig. 1 shows that by the end of this time the fat content had been reduced to a very low level, being 2.5 and 2.4 per cent for germination in the light and dark, respectively. The sterol content, on the other hand, showed an increase of 93 and 101 per cent, respectively. However, the increase during this period of growth was again limited chiefly to the free sterol.

<div align="center">DISCUSSION</div>

The two varieties of soy bean (mammoth yellow and black Wilson) were used since it seemed of interest to determine whether their marked difference in color was reflected in their fat composition. Apparently such was not the case, the values for the ungerminated seeds of the two varieties being much the same. This lack of relationship between physical characteristics of different varieties of seeds and their fat composition was also observed by Guerrant (1927) in the case of two varieties of grain sorghum which differed widely in both size and color.

The decrease in fat content as germination proceeds is a common observation and is in accordance with the observations on oleaginous seeds by Pierce, Sheldon, and Murlin (1933) and by numerous other investigators. Undoubtedly the fat serves as a reserve of food for the growing plant. Why the decrease in fat was more marked in the light than in dark during the 2nd week of germination is not clear. It was, however, a constant finding.

On the other hand, there was a continued synthesis of sterol throughout germination in both the light and the dark. Usually it was slightly greater during germination in the dark than in the light. This is in agreement with the findings of Beumer (1933). However, Beumer's explanation that it is related to the greater growth intensity or cell formation in the dark is difficult to accept since the plants in the light grew as high if not higher than those

in the dark. Certainly the dry weight of the normal plants was greater, which means that there was a greater amount of cell formation.

Miller (1910, 1912) observed in the case of the sunflower that although the total fat decreased considerably during the 1st week of germination, the percentage of free fatty acids was very low. During the 2nd week the fat continued to decrease but there was a marked liberation of free fatty acids such that by the 14th day of germination they had attained a value of 30 per cent or more. These observations might account for the lack of esterification of the sterol which we have noted (Fig. 1) during the 1st week of germination of the soy bean and the marked esterification of the sterol during the 2nd week of germination. The slightly greater production of ester sterol in the dark may be related to the higher per cent of fat and therefore the greater abundance of free fatty acids.

According to Miller (1931) the per cent of fat in soy bean stems and leaves is 2.5 per cent. Consequently the fact that during the 3rd week of germination the increase in sterol is again confined chiefly to the free sterol may be due to the fact that the fat content has reached its minimal level; i.e., that there is a relative paucity of free fatty acids.

In short we may say then that there is a continuous synthesis of sterol in seedlings during germination in both the light and the dark, and that during the period of intense fat mobilization and utilization there is a marked esterification of the sterol.

It has been shown by Knudson (1917) that in dogs, after the ingestion of fat, the ratio of cholesterol ester to free cholesterol in the blood rises. Again Bloor (1932) demonstrated in the same animals that the per cent of the total cholesterol present as ester is always higher on a high fat diet than on a low fat diet. It would appear then that the close relation found to exist between the esterification of sterol and the utilization of fat in plants is much the same as that observed in certain animals (dog).

SUMMARY

1. The changes in the sterol relative to those in the total fatty acids were determined in mammoth yellow and black Wilson soy beans before and after germination in the light and in the dark.

It was found that (a) while the total fat diminished markedly as germination proceeded, there was a continuous synthesis of sterol which was somewhat greater during growth in the dark than during growth in the light, and (b) during the period of rapid fat mobilization and utilization, there was a marked esterification of the sterol.

2. The results suggest that there is a close relation between the sterol metabolism and the fat metabolism in plants much the same as in animals.

The author takes pleasure in acknowledging his indebtedness to Professor W. R. Bloor for advice and helpful criticism throughout this investigation.

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THE EFFECT OF THE INGESTION OF COTTONSEED OIL BEFORE AND AFTER HYDROGENATION ON THE COMPOSITION OF THE BODY FAT OF THE RAT ·

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· Contrary to the usual softening influence of ingested plant oils upon animal body fats, cottonseed oil, within certain limitations, has been found to produce hard fats. Much of the work has been done with hogs, in the case of which the firmness of the lard produced is an important economic consideration. Ellis and Isbell (1) showed that while 4 per cent additions of soy bean, corn, and peanut oils to their basal diet changed the fat constants of the resulting body fat in the direction of greater unsaturation and softness, the addition of cottonseed oil at the same level produced the opposite effect.

In subsequent work (2) it was found that an increase in the cottonseed oil content of the diet to 8 per cent somewhat reduced the hardening effect, while 12 per cent produced softening of the body fat. Analyses of the fatty acid composition of the body fats with particular attention to the changes in the percentages of oleic, linoleic, stearic, and palmitic acids revealed a persistent reduction in oleic and an increase in stearic over the usual values. In an attempt to determine whether the hardening property resided in the liquid or solid fraction as obtained in the lead salt-ether separation of fatty acids, rats were fed these fractions as well as the whole oil at different levels. No effort was made to fractionate the body fats of the rats, and the results of fat-constant determinations failed to reveal any pronounced changes which would be attributed to either the liquid or the solid fraction.

However, the results on the hog fats appeared to indicate that

the linoleic and oleic acids of the liquid fraction were involved presumably through their ready conversion into stearic acid. Whatever the nature of the steps involved, they at least suggested that structural changes in component fatty acids must have taken place during metabolism to modify the deposited fat to the extent noted.

The present paper gives some results of further experiments on the effect of the ingestion of cottonseed oil upon the composition of the body fat with the rat as the experimental animal. For want of a complete separation of the oil into fractions of pure fatty acids for feeding purposes, preferential hydrogenation was selected as the agent to effect changes in the natural oil. The three samples made available for feeding purposes all originated from the same batch of refined oil and consisted of the original unhydrogenated oil and two hydrogenated samples which differed with respect to their type of hydrogenation.

Rats were used because of the limited amount of specially prepared fat which could be made available for feeding. Furthermore, information as to the effect of ingested fat upon the composition of the body fat of the rat is meager. Banks, Hilditch, and Jones (3) have applied the ester fractionation method in their studies on the component fatty acids in the adipose tissue of rats fed on diets varying in fat content. The presence of palmitoleic acid as a normal component of the depot fat of the rat was suspected by these investigations and has been confirmed more recently by Klenk, Ditt, and Diebold (4).[1]

<center>EXPERIMENTAL</center>

Male albino rats were placed on the experimental diet when 4 weeks old and fed individually for a period of 10 weeks. Four groups of six animals each were used. One group, Group A, which served as a control was fed the basal diet which contained only a small amount of preformed fat naturally present in the dietary constituents. This basal diet consisted of casein 18 per cent, dextrin (from corn-starch) 64 per cent, agar 1 per cent, salt mixture[2]

[1] This publication appeared while the present work was in progress.
[2] The salt mixture as used for a number of years has been described by another member of the laboratory staff of the division (Barnum, G. L., *J. Nutrition*, **9**, 624 (1935)).

4 per cent, alfalfa leaf meal 5 per cent, yeast (including an irradiated portion) 8 per cent. Vitamin A was supplied in addition in the form of a non-saponifiable fraction of cod liver oil. The other groups were fed this basal diet with the three fats under investigation incorporated at an 8 per cent level by replacement of an equal amount of dextrin. Group B received the non-hydrogenated oil (Fat I); Group C, the oil (Fat II) which was hydrogenated to reduce moderately the linoleic acid content; and Group D, the oil (Fat III) hydrogenated for maximum reduction of this acid.

At the end of the experimental period the animals were weighed, then killed, and, after the carcasses were chilled by refrigeration, the adipose tissue was removed and combined by feeding groups.

TABLE I

Composition of Dietary Fat

Dietary fat No.	Iodine No.	Thio-cyanogen No.	Fatty acids in fat				
			Saturated	Linoleic	Oleic + isoleic	Isoleic	
						Twitchell method	C—C—H
			per cent	per cent	per cent	per cent	per cent
I	107.93	64.15	28.8	48.5	22.7	0.2	6.7
II	68.79	57.97	35.5	12.0	52.5	13.8	23.8
III	60.20	58.10	35.4	2.3	62.3	14.9	31.5

The total fatty acids were obtained from the four groups by saponification and recovery by ether extraction from the acidified saponification product.

The subsequent analyses of the component fatty acids were carried out by the ester fractionation method essentially as used by Hilditch and Jones (5). The methyl esters of both the solid and liquid fractions were prepared by refluxing with an excess of methyl alcohol containing 4 per cent of sulfuric acid. In the case of the liquid acids carbon dioxide or nitrogen was passed through the reaction flask during esterification. The methyl esters after purification in the usual manner were fractionally distilled from a flask with an electrically heated column designed by Klenk and Schoenebeck (6) for work of this kind. Saponification values and iodine numbers were then determined on each fraction with

thiocyanogen numbers in addition on the liquid acid fractions and the necessary calculations made in order to obtain the amounts present for each acid.

The per cent of oleic, of linoleic, and of total saturated acids in the cottonseed oil samples used in the diets were also calculated by means of appropriate equations (7) from the iodine and thiocyanogen numbers determined on the samples. The percentage of isooleic acid was obtained by the Twitchell (8) as well as the Cocks, Christian, and Harding method (9) on both the dietary fat and the body fatty acids.

Results

Composition of Dietary Fats—From the analyses given in Table I on the composition of the oils used in the diets, it is apparent that

TABLE II

Yields and General Composition of Body Fat of Rats

Group	Average weights			Iodine No.	Mean mol. wt.	Lead salt separation on fatty acids	
	Rats	Adipose tissue	Total acids			Solid	Liquid
	gm.	*gm.*	*gm.*			*per cent*	*per cent*
A	316	45.1	30 6	59.65	264.1	41.2	58 8
B	308	33.0	19.9	83 85	270.1	39.2	60.8
C	308	41.6	28 1	69.83	268 0	46 5	53.5
D	282	30.2	19.5	64.79	274.7	46.7	53 3

the hydrogenation of cottonseed oil resulted in the conversion of most of the linoleic into oleic acid before the double bond of the latter was appreciably affected. Thus, in comparison with the untreated oil, Fat III showed a drop in thiocyanogen number of only 6.05 units compared to a drop of 47.74 units in iodine number. This corresponds to a drop of 46.2 in the percentage of linoleic acid as compared to an increase of 6.7 in the percentage of the total saturated acids.

The data in Table I also show that the hydrogenation process resulted in the formation of a considerable amount of isooleic acid. A wide difference in the actual amounts given by the two methods is apparent from the results for all three fats. The Twitchell

method gave a maximum of 14.9 per cent as compared to 31.5 per cent by the Cocks, Christian, and Harding method.

Analyses of Body Fats—Table II records the data on the growth of rats in each group, the yields of adipose tissue, certain fat constants, and the separation into solid and liquid acids. It will be noted that the animals on the low fat basal diet made the highest gains in weight. Separation of the liberated fatty acids from the four composite samples of the adipose fat into solid and liquid fractions by means of the Twitchell method gave yields of liquid acids from the groups receiving the hydrogenated oils only 0.2 per cent apart and lower than either the low fat or the natural oil groups.

Table III gives the weights, iodine numbers, and mean molecular weights (calculated from saponification values) of the fractions resulting from the distillations. In the analyses of the esters of the solid acid fractions each ester fraction was assumed to contain one or, at the most, two homologous saturated acid esters, and isooleic as the unsaturated acid ester. The acids found present were myristic, palmitic, stearic, small amounts of acids of higher molecular weight than C_{18} acids, and isooleic acid.

Examination of the liquid acid esters after fractional distillation indicated the presence of methyl palmitoleate, methyl oleate, and methyl linoleate besides small amounts of saturated acid esters in Groups B, C, and D. Linoleic acid was apparently absent in the fat of Group A. In order to calculate the amounts of the individual acids in such a four component system as present in Groups B, C, and D, it was necessary to assume that the oleic and linoleic esters distilled over in a constant ratio just as is done in the three component system containing only oleic and linoleic esters as unsaturated components. Calculation of the fourth component, namely the palmitoleate, is made possible by the use of the thiocyanogen number. From the iodine and thiocyanogen numbers on the fractions the weights of the component acids were calculated by means of the following equations in which x represents the weight of methyl oleate; y, the weight of methyl palmitoleate; z, the weight of methyl linoleate; s, the weight of saturated esters; wt, the weight of the methyl fraction; IN, the iodine number of the methyl fraction; TN, the thiocyanogen number of the fraction; in, the iodine number of the pure C_{18} fraction; SV, the saponification value of the fraction.

$$(1) \qquad x + y + z + s = wt$$

$$(2) \qquad 0.9461x + 0.8567y + 1.762z = (IN/100)\ wt$$

$$(3) \qquad 0.9461x + 0.8567y + 0.8568z = (TN/100)\ wt$$

$$(4) \qquad \frac{y\ 172.6 - in}{-85.67}\ K$$

TABLE III

Fractional Distillation of Methyl Esters

Group	Solid acid fraction				Liquid acid fraction				
	Fraction No.	Weight	Mean mol. wt.	Iodine No.	Fraction No.	Weight	Mean mol. wt.	Iodine No.	Thio-cyanogen No.
		gm.				*gm.*			
A	1	10.498			1	1.611	265.3	71.66	66.73
	1-a	2.830	256.3	4.79	2	2 329	276.2	83.62	83.18
	1-b	3.722	267.4	4.95	3	2.552	282.9	85.50	84.47
	1-c*	3.814	271.8	7.63	4	3.118	292.0	85.80	84.44
	2	8.097	269.7	6.53	5	2.901	295.0	85.55	84 90
	3	5.726	270.9	7.25	6*	2.275	(296.8)	(83.90)	
	4	8.313	271.5	8.50		14.786†			
	5	3.851	273.4	13.46		15.034§			
	6	3.526	279.1	22.37					
	7	3.685	289.9	39.33					
	8*	2.857	297.2	43.05					
		46.554†							
		46.754‡							
B	1	2.327	264.4	1.89	1	2.760	265.8	85.57	55.00
	2	2.816	268.1	1.27	2	3.885	282.9	110.21	77.50
	3	2.831	270.7	1.25	3	4.715	287.8	119.90	81.09
	4	3.673	271.7	1.27	4	5.224	292.0	128.03	83.45
	5	3.654	270.9	1.58	5	3.421	294.3	131.35	85.00
	6	4.086	271.4	1.61	6	8.840	294.9	132.16	86.30
	7	3.255	270.6	2.46	7	3.342	295.5	131.80	
	8	1.907	271.2	3.75	8	2.235	296.5	131.07	
	9	3.536	279.5	7.26	9*	5.312	(298.8)	(110.15)	
	10	1.699	287.3	12.47		39.734†			
	11*	2.130	291.8	27.37		40.078‡			
		31.914†							
		31.973‡							

* Residue.

† Weight recovered.

‡ Weight distilled.

§ Weight distilled. Unfortunately some of the sample was lost during the preparation of the methyl esters.

TABLE III—*Concluded*

Group	Solid acid fraction				Liquid acid fraction				
	Fraction No.	Weight	Mean mol. wt.	Iodine No.	Fraction No.	Weight	Mean mol. wt.	Iodine No.	Thiocyanogen No.
		gm.				*gm.*			
C	1	11.240			1	12.240			
	1-a	3.437	259.1	6.20	1-a	3.024	258.0	65.73	62.35
	1-b	3.426	268.3	7.71	1-b	2.890	·273.7	85.35	80.52
	1-c	3.236	272.8	13.18	1-c	3.406	280.9	91.38	82.96
	1-d*	1.129	283.9	37.18	1-d*	2.851	296.2	99.92	86.18
	2	8.008	273.4	15.51	2	5.925	285.7	94.82	84.30
	3	6.335	274.6	19.70	3	6.755	292.0	98.66	84.83
	4	5.360	277.3	27.32	4	6.565	293.3	101.0	85.60
	5	5.603	283 0	39.93	5	9.544	295.7	102.0	86.19
	6	5.012	290.8	59.75	6	5.092	295.8	102.6	85.70
	7	4.106	294.0	66.37	7	3.196	296.0	102.4	
	8*	1.321	299 5	64.41	8	3.045	296.1	103.0	
		46.985†			9*	2.911	(298.8)	(97.88)	
		47.112‡				55.273†			
						55.551‡			
D	1	6.950			1	2.168	269.0	73.37	70.38
	1-a	1.179	257.1	1.01	2	3.002	279.5	85.31	82.22
	1-b	1.186	264.0	6.34	3	4.603	286.1	88.20	84.00
	1-c	3.723	270.3	9.48	4	3.294	289.7	90.64	84.87
	1-d*	0.872	276.0	26.00	5	4.527	293.6	91.75	85.48
	2	5.478	272.7	14.60	6	7.031	295.1	92.53	85.86
	3	8.600	275.5	21.27	7*	2.071	(299.1)	(99.90)	
	4	6.180	279.0	28.10		26.696†			
	5	4.663	285.2	43.22		26.862§			
	6	5.956	293.4	62.90					
	7*	1.142	300.9	62.50					
		38.969†							
		39.192‡							

By combining Equations 2 and 3 one gets

$$(5) \qquad z = \frac{(IN - TN)\, wt}{86.9}$$

From Equations 4 and 5 one gets

$$(6) \qquad y = Kz = \frac{K(IN - TN)\, wt}{86.9}$$

Substituting in Equation 2 the values for y and z, one obtains

$$(7) \qquad x = \frac{(IN/100)\, wt - 0.8567y - 1.726z}{0.9461}$$

and finally

(8) $$s = wt - x - y - z$$

The saturated acid found by this method of calculation was assumed to be myristic acid, although traces of other acids were undoubtedly present.

Calculations of the component acids in the fractions resulting from the distillation of the liquid acids from Group A, in which linoleic acid was absent or nearly so, were carried out by a different set of equations with the data on saponification values and iodine numbers. The equations follow:

(1) $$x + y + s = wt$$

(2) $$0.9461x + 0.8567y = (IN/100) \; wt$$

(3) $$209.13x + 189.36y + 231.57s = SV \; wt$$

From Equations 2 and 3, is obtained

(4) $$s = \frac{wt(SV - 2.2104) \; IN}{231.57}$$

When the value for s is substituted in Equation 2,

(5) $$x = \frac{(IN/100) \; wt - 0.8567 \; (wt - s)}{0.0894}$$

and by difference,

(6) $$y = wt - x - s$$

In the ester fractionation data, recorded in Table III, several points of interest are brought out. The assumption was made in one of the calculations described above that the methyl esters of oleic and linoleic acid distilled from a mixture in a constant ratio. Evidence in support of this assumption is to be found in the data on Fractions 5 to 8 of the liquid acid esters from Group B. The constancy of iodine numbers and mean molecular weights indicates that methyl oleate and methyl linoleate distilled in a definite ratio in all these fractions.

A similar result is to be observed on examining the distillation data for the liquid esters from Group C. The fact that the ratio is different in each group rules out the probability of a constant

boiling mixture and makes it appear very probable that the ratio in which they appear in the distillate depends upon the ratio of the two in the mixture distilled.

Justification for the use of the thiocyanogen number in conjunction with the iodine number for purposes of analysis is to be found in the results obtained in the distillation of the liquid esters.   In each case where linoleic acid was a constituent of the dietary fat, its presence in the corresponding adipose fat was indicated by the difference between the iodine number and the thiocyanogen number of the liquid ester fractions.

In the case of the esters from the fat of the rats on the low fat diet, these two constants were sufficiently close to one another to preclude the possibility of any appreciable amount of linoleic acid being present.   This fact was substantiated by the iodine number of the pure C_{18} acid fraction, which was very close to that required for methyl oleate.

Evidence that linoleic acid absorbs only 1 mole of thiocyanogen is also to be found in the distillation data.   In those liquid ester fractions where the molecular weight indicates that only methyl oleate and methyl linoleate are present, the thiocyanogen number is very close to 85.6, the theoretical value for a mixture of these two esters.

The presence of an acid with a molecular weight higher than that required for a C_{18} acid was indicated by the mean molecular weights of the residues obtained from the distillation of the liquid esters of rats fed on fat-containing diets.   In order to confirm this observation the liquid ester residues of all four rats were brominated in ether solution.   All three residues from the rats on fat-containing diets gave an ether-insoluble bromide which was also insoluble in boiling benzene.   This bromide had no melting point but blackened sharply at 233–235° in accordance with the usual behavior of arachidonic acid.   After bromination of the residue from the fat from Group A, however, no ether-insoluble residue was obtained.

Since it was thought that the presence of arachidonic acid in the adipose fat might be due to its presence in the dietary fat, bromination experiments were performed on the latter, but no trace of an ether-insoluble bromide was found.

Although the presence of palmitoleic acid in the adipose fat of

rats was first suspected by Banks, Hilditch, and Jones (3), their evidence was indirect and inconclusive.   More conclusive but still indirect evidence is to be found in the distillation data in Table III.   Thus, the difference between the iodine number and the thiocyanogen number of the early liquid ester fractions of low molecular weight can be most satisfactorily explained by the presence of a single bond acid with a molecular weight lower than that required for a C_{18} acid.

An attempt to provide more conclusive evidence for the presence of this acid by actually isolating and identifying it was only partially successful.   The method employed was based on the observation that its dibromide could be distilled at low pressure without decomposition.   Several primary liquid ester fractions were combined and brominated in ether solution.   Any insoluble bromides were filtered off, and the other solution of bromides was freed of bromine by washing with thiosulfate solution and then water.   The ether solution was dried over anhydrous sodium sulfate and after removal of the solvent, the brominated esters were distilled *in vacuo*.   The saturated acid esters were collected in the first fraction which distilled at 130–135°, at 2.5 mm.   The temperature then rose rapidly to 195°, and a second fraction was collected at 195–200°.   It was slightly yellow in appearance.   This fraction was reduced by refluxing with zinc dust and methyl alcohol.   The recovered unsaturated ester in ether solution was washed with dilute hydrochloric acid, then water, and finally dried.   After reesterification, the methyl ester was distilled.   The main portion came over at 125–129° at 1.8 mm.   It had an iodine number of 92.5 and a molecular weight of 270.0 (theory for methyl palmitoleate, iodine number 94.6, mol. wt. 268.3).

The effect of ingested fat on the composition of adipose fat is illustrated in the summarized data given in Table IV.   The chief differences, it will be noted, occur among the unsaturated components.   The saturated acids, on the other hand, show no such wide variations.   However, it will be observed that the highest values for palmitic acid and stearic acid were obtained with Group B which was fed the most unsaturated fat.

Although no extreme accuracy is claimed for the analyses reported, owing to the nature of the methods involved, it is felt that they are of sufficient accuracy to justify the conclusions drawn.

In general, the results reported are in accordance with the results obtained in the two investigations already mentioned even though different methods of analysis were used in each case.

TABLE IV

Component Fatty Acids of Body Fat

Group	Myris-tic	Pal-mitic	Stearic	Higher than C₁₈	Total satu-rated acids	Palmit-oleic	Oleic + iso-oleic	Lino-leic	Arachi-donic	Total unsatu-rated acids
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A	5.6	29.3	2.5	0.0	37.4	14.0	48.6	0.0	0.0	62.6
B	3.4	32.8	3.4	0.0	39.6	2.0	30.2	27.3	0.9	60.4
C	4.6	25.8	1.6	0.2	32.2	6.8	52.3	8.3	0.4	67.8
D	4.3	26.4	2.0	0.2	32.9	8.8	54 4	3.3	0.6	67.1

TABLE V

Determination of Isooleic Acid Content of Body Fat

Group	Method	Solid acid frac-tion	Iodine No.	Mean mol. wt.	Liquid acid frac-tion	Iodine No.	Mean mol. wt.	Isooleic in solid fraction	Isooleic in original acids
		per cent			*per cent*			*per cent*	*per cent*
A	Twitchell	34.5	1 67	261.0	64.3	88.87	272.4	1.9	0.7
	C—C—H	41.7	17.12	258.6	54.7	88.10	273.8	19.0	8.2
B	Twitchell	35.8	1.64	265.3	61.5	124.1	275.0	1.8	0.7
	C—C—H	38.2	7.17	256.9	57.7	120.7	270.5	8.0	3.2
C	Twitchell	33.8	12.52	263.4	60.6	98.16	277.5	13.9	5.0
	C—C—H	49.3	32.85	260.6	47.5	96.70	274.1	36.5	18.6
D	Twitchell	30.6	10.28	261.9	65.2	89.04	278.1	11.4	3.6
	C—C—H	51.1	35.00	264.0	45.9	91.87	277.0	38.9	20.5

DISCUSSION

The question as to whether or not the rat can synthesize linoleic acid has been raised by several investigators since Burr and Burr (10) showed that this acid was a factor essential to growth and well being. It was their contention that the rat could not synthesize linoleic acid and some other highly unsaturated acids. The observations of Sinclair (11), however, were opposed to this view. Furthermore, Eckstein (12) succeeded in demonstrating the presence of linoleic and arachidonic acids in the body lipids of rats fed on diets devoid of these acids. The results of an investi-

gation by Gregory and Drummond (13) tend to reconcile these two points of view.   They concluded from a study of the analytical constants of the liquid acid fractions obtained by the Twitchell method from depot and liver fat that the rat could synthesize linoleic acid but not store it in its depot fat.   This was confirmed in part by the work of Banks, Hilditch, and Jones (3), who concluded from their study of the adipose fat of rats that this animal did not store any appreciable amount of linoleic acid.   Further confirmatory evidence for this fact is presented as a result of the present investigations.

Although the presence of linoleic acid could not be demonstrated in the adipose fat of rats fed on the low fat diet, its presence was easily detectable when it was present in the diet to the extent of only 0.2 per cent.   The readiness with which this acid is deposited naturally raises the question as to the minimum amount necessary for well being.

Another acid whose wide-spread occurrence in body tissue of various animals has led to the belief (14) that its presence is necessary for cellular activity is arachidonic acid.   Although Eckstein (12) was able to demonstrate its presence in different phospholipid-containing fractions of rat body fat, the work of Banks, Hilditch, and Jones (3), which is also confirmed by the data herein reported, indicates that it is not a normal component of stored fat.   In the present investigation, to be sure, its presence was detected in the depot fat of rats fed fat-containing diets even though bromination experiments failed to reveal any trace of it in the ingested fat.

However, this discrepancy can be explained if one bears in mind the fact that a small amount of arachidic acid (usually less than 1 per cent) is present in cottonseed oil and, hence, also in its hydrogenated products.   Furthermore, as Barbour (15) has shown, the rat has a very low tolerance for this acid.   It appears, therefore, that not only is the ingestion of arachidic acid by the rat followed by its deposition in the body fat in small amounts, but a dehydrogenation of this acid to arachidonic acid with a subsequent deposition of the latter also takes place.   The dehydrogenation process is undoubtedly the result of an attempt by the rat to convert the high melting arachidic acid into a more easily mobilized liquid acid. No satisfactory explanation can be offered for the failure to find a saturated acid of higher molecular weight than C_{18} in the saturated

acid fraction obtained from the rats fed the cottonseed oil-containing diet.

The feeding of cottonseed oil to rats gave results similar to those obtained by the feeding of this oil to hogs. Thus the rats fed diets containing cottonseed oil deposited the largest amounts of saturated acids in their body fats. Although the differences for the saturated acid content of the stored fat obtained in the present series of experiments were small, they may, nevertheless, be regarded as significant.

In accordance with the results obtained by Barbour (16), it was found that the ingestion of isooleic acid resulted in its deposition in the depot fat.

SUMMARY

The normal acid components of the body fat of the rat were found to be myristic, palmitic, stearic, palmitoleic, and oleic acids.

The ingestion of fats of varying degrees of unsaturation affected the composition of the saturated acid fraction of the body fat to a less extent than it did the unsaturated acid fraction. The rats fed cottonseed oil deposited more palmitic and stearic acids than those fed partially hydrogenated cottonseed oil or the low fat diet.

The ingestion of linoleic acid resulted in its deposition in the adipose fat. The amount deposited was approximately proportional to the amount ingested. Increased deposition of linoleic acid was accompanied by a decreased deposition of palmitoleic and oleic acids. The body fat of rats fed cottonseed oil or hydrogenated cottonseed oil contained small amounts of arachidonic acid and a saturated acid of higher molecular weight than C_{18}. Ingested isooleic acid was deposited in the depot fat.

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THE COMPONENT FATTY ACIDS OF GOAT MILK FAT

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Milk fats from different species of mammals are known to vary considerably in chemical composition. In early work, the butter fat constants were largely used to indicate the differences in composition. More recently, emphasis has been placed on comparisons of the component fatty acids of the various types of butter fats. The methods available for separation of the acids are not conducive to high precision. Their lack of uniformity, their dependence upon the manipulative technique, and upon the refinement of equipment have led to contradictory results and to differences of opinion as to the presence of certain acids.

The distillation of the methyl esters is the most widely used method of effecting the separation of the components of butter fats. By the employment of this method and subsequent analyses of the distilled fractions, the presence of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, and oleic acids in the fat of cow's milk has been established without serious question. However, from time to time, other acids have been reported. Smedley (1), in 1912, inferred the presence of a C_{10} unsaturated acid after he obtained a maximum in the iodine number of a C_{10} fraction in his distillation data. Crowther and Hynd (2), in 1917, on the other hand, attributed the unsaturation of the lower fractions of butter esters to be due to oleic acid. Grün and Wirth (3), in 1922, isolated decenoic acid from the lower boiling acids and established the position of the double bond as the (9, 10) position. Bosworth and Brown (4), in 1933, made a detailed study of the component acids of butter fat of cow's milk in which they, too, found decenoic acid. In addition they presented evidence indicating the presence of a number of other acids; namely, tetradecenoic acid, a C_{20}, C_{22},

or C_{24} acid containing two double bonds, acids of the arachidonic type probably belonging to the C_{22} series and a mixture of saturated acids of high molecular weight consisting principally of tetracosanoic acid and probably small amounts of behenic and cerotic acids. However, Hilditch and coworkers (5, 6) have reported, in addition to the commonly accepted acids, linoleic and arachidic acids. Eckstein (7) also reported the presence of linoleic acid and added linolenic acid to the list of disputed acids.

Comparatively little work has been done on the composition of goat milk fat. Dhingra (8), in 1933, using the procedure developed by Hilditch and coworkers (5, 6) reported the same acids present in goat milk fat as were reported by the latter in cow's milk fat. The question of certain unusual characteristics in the fat of goat milk, which were concerned in the development of anemia in infants, has been suggested by a number of workers. De Rudder (9) has intimated that certain acids in goat milk fat may have a hemolytic action, producing anemia in infants, and Stoltzner (10) has stated that the high content of volatile acids has a definite effect upon anemia development. However, recent investigations,[1] of which the present report has formed a phase, have shown no differences between goat and cow's milk in their hemoglobin-generating properties.

Human milk fat was found by Bosworth (11) to differ considerably in chemical composition from cow's milk fat in that it contained linoleic and palmitoleic acids and very little of the volatile acids from butyric to capric. Although Bosworth did not isolate palmitoleic acid in pure form, he obtained evidence that indicated its presence. Since this acid has not been reported in the fat of cow's or goat milk, there is a possibility that its presence may have been overlooked.

Lintzel has reported evidence (12) which shows that milk fat is produced in the mammary gland from glycerides taken from the blood stream. The question arises concerning the source of these glycerides, their precursors, and mode of formation. The fact that body fats are quite simple in comparison to butter fats and

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[1] Other phases of these investigations bearing on the nutritive value, composition, and physical properties of goat milk with comparisons to cow's milk are in progress of publication as a technical bulletin of the United States Department of Agriculture.

can be greatly influenced by the character of the feed naturally gives rise to the question of how greatly the nature of the fatty acids of milk fat can be changed by the diet of the lactating animal.

The present paper reports the results of a study on the nature and composition of a composite sample of fat prepared from the milk of a goat herd kept on a regulated dietary regimen. Attention was given to the identification and estimation of the approximate amounts of each fatty acid normally present, especially the disputed acids. The findings of Bosworth and Brown (4) on the presence of hitherto unreported and the absence of certain other fatty acids in cow's milk fat have prompted the use of their procedure in the present investigations. Their procedure differs from that used by Hilditch and coworkers (5, 6) in that the separation .into liquid and solid acids is not made prior to the distillation of the methyl esters. The extensive series of distillation fractions obtained directly from the mixed esters enables a thorough examination of the individual fractions.

<center>EXPERIMENTAL</center>

Source of Sample—The sample of goat milk fat consisted of 3.5 kilos of filtered butter fat obtained from a herd of twenty-four Saanen and Toggenberg does. Cream was obtained periodically from May to October, 1934, and churned while sweet. The portions of filtered butter fat were stored in a refrigerator until the required amount was collected. The goat does received a ration consisting of 1.5 pounds per day of a grain mixture of 8 parts corn, 4 parts oats, 2 parts wheat bran, and 1 part linseed meal, 3 pounds per day of alfalfa hay, and pasturage. The pasturage during May consisted of wheat and barley and thereafter of permanent pasture with access to an abundant growth of browse. From June 1 to July 15 the grazing period was limited to ½ hour per day and thereafter unlimited except for milking periods.

Water-Soluble Acids—After saponification of the butter fat, the water-soluble acids were recovered and estimated by a procedure similar to that used by Hilditch and Jones (5). A total of 68.8 gm. of butyric and 7.5 gm. of caproic acids was accounted for from the 3.5 kilos of butter fat.

Preparation and Distillation of Methyl Esters of Insoluble Acids— The water-insoluble fatty acids were esterified with methyl alcohol

and dry HCl.   These methyl esters were then divided into three approximately equal portions and each portion was distilled into thirteen fractions, including the residue.   Corresponding fractions from each portion were combined.   The first ten of these combined primary fractions were redistilled, the residue from the lower boiling fraction always being added to the succeeding fraction. Since some decomposition and polymerization usually takes place during the distillation of the higher unsaturated esters, the last three of the combined primary fractions were not redistilled.   The distillations were carried out in a specially constructed electrically heated still similar in design and size to that described by Bush and Schwartz (13).   An open tube column 10 mm. in diameter was used.   A spiral of nichrome wires containing about 125 turns, wound so as to fit snugly within the walls of the column, and a smaller spiral of about the same number of turns wound in the opposite direction were employed to decrease the "channeling" of the vapors.   A 100 cc. Bogert receiver was used to collect the primary fractions, while in the redistillation a Brühl receiver capable of collecting seven 50 cc. fractions was employed.   The data from the 63 fractions obtained are given in Table I.

Examination of Distillation Data—The mean molecular weights of Fractions 1 to 3, 6 to 9, 14 to 18, 21 to 22, 26 to 28, 40 to 49, and 57 to 62 indicate the presence of C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} saturated acids.   From the constancy of the iodine numbers of the fractions mentioned, the presence of C_{10}, C_{14}, C_{16}, and C_{18} unsaturated acids seems likely.   To indicate this more clearly a curve plotting iodine absorption of the fractions against their mean molecular weights was drawn.   Those fractions whose mean molecular weights were nearly the same were treated as one fraction.   The curve constructed accordingly is shown in Fig. 1.   Definite peaks of iodine absorption occur at the mean molecular weights of C_{10}, C_{14}, C_{16}, and C_{18} acid esters.   The fractions of methyl esters were subjected to further study to obtain more definite proof of their fatty acid constituents.

Decenoic Acid—Fractions 4 to 17, after bromination in ether, were fractionally distilled, a Claisen flask with the side arm modified to form a short electrically heated column insulated with asbestos cement being used.   As shown in Table II, the analyses of the bromide obtained correspond to those of methyl dibromocap-

TABLE I

Data Obtained from Distillation of Methyl Esters of Mixed Fatty Acids and Analyses of the Fractions

Fraction No.	Weight	B.p.	Pressure	Acid series	Mean mol. wt.	I No.
	gm.	*°C.*	*mm.*			
1	17.5	75– 77	75	C_6	130.7	0.2
2	17.7	77– 79	75	C_6	130.7	0.2
3	16.9	79– 83	75	C_6	130.8	0.2
4	8.1	84– 89	75	C_6–C_8	134.2	0.7
5	16.6	90–114	75	C_6–C_8	149.5	0.8
6	16.3	114–116	75	C_8	156.2	0.4
7	15.3	116–119	75	C_8	156.4	0.3
8	13.7	119–120	75	C_8	158.1	0.6
9	12.4	120–125	75	C_8	159.6	0.5
10	13.6	126–132	75	C_8–C_{10}	161.8	0.7
11	12.2	132–140	75	C_8–C_{10}	167.8	1.4
12	11.2	140–144	75	C_8–C_{10}	173.8	2.5
13	11.7	144–150	75	C_8–C_{10}	181.4	2.6
14	44.0	81– 82	1–2	C_{10}	183.8	2.0
15	46.1	82	1–2	C_{10}	184.6	2.2
16	42.1	82	1–2	C_{10}	185.4	3.1
17	47.2	82– 84	1–2	C_{10}	186.0	3.3
18	42.0	84– 89	1–2	C_{10}	188.0	3.2
19	6.2	90– 91	1–2	C_{10}–C_{12}	192.0	3.5
20	46.7	91– 97	1–2	C_{10}–C_{12}	203.8	3.4
21	50.8	98–103	1–2	C_{10}–C_{12}	210.5	3.5
22	41.7	103–117	1–2	C_{12}–C_{14}	218.7	3.7
23	41.1	119–120	1–2	C_{12}–C_{14}	231.4	3.2
24	30.6	120	1–2	C_{12}–C_{14}	235.6	3.9
25	40.5	120–123	1–2	C_{12}–C_{14}	237.5	3.8
26	42.6	123	1–2	C_{14}	241.3	3.0
27	37.9	123–124	1–2	C_{14}	241.5	3.5
28	37.2	124	1–2	C_{14}	241.7	4.7
29	40.5	124–126	1–2	C_{14}–C_{16}	244.4	4.0
30	28.8	126	1–2	C_{14}–C_{16}	247.3	4.1
31	40.3	126–130	1–2	C_{14}–C_{16}	252.0	5.9
32	34.7	130–136	1–2	C_{14}–C_{16}	257.2	5.7
33	40.3	136	1–2	C_{14}–C_{16}	260.6	6.0
34	43.6	136	1–2	C_{14}–C_{16}	263.6	6.9
35	40.2	136	1–2	C_{14}–C_{16}	263.8	7.0
36	43.7	136	1–2	C_{14}–C_{16}	266.1	7.3
37	39.1	136	1–2	C_{14}–C_{16}	266.5	6.7
38	46.3	136	1–2	C_{14}–C_{16}	267.6	6.8
39	43.5	136	1–2	C_{14}–C_{16}	267.9	6.9

TABLE I—*Concluded*

Fraction No.	Weight	B.p.	Pressure	Acid series	Mean mol. wt.	I No.
	gm.	*°C.*	*mm.*			
40	47.4	136	1-2	C_{16}	268.9	6.3
41	42.1	136	1-2	C_{16}	269.1	7.4
42	44.3	136	1-2	C_{16}	269.2	7.4
43	41.8	136	1-2	C_{16}	269.8	7.9
44	48.9	136–138	1-2	C_{16}	270.3	8.7
45	45.1	138–140	1-2	C_{16}	270.3	10.2
46	47.9	140–142	1-2	C_{16}	270.4	7.8
47	52.0	142	1-2	C_{16}	270.4	8.9
48	42.2	142	1-2	C_{16}	271.4	10.3
49	42.9	142	1-2	C_{16}	271.6	12.0
50	44.3	142–145	1-2	C_{16}–C_{18}	272.6	14.3
51	40.7	145	1-2	C_{16}–C_{18}	273.6	18.0
52	39.4	145	1-2	C_{16}–C_{18}	275.1	19.9
53	43.6	145–150	1-2	C_{16}–C_{18}	279.1	32.4
54	42.7	150	1-2	C_{16}–C_{18}	280.2	38.0
55	44.3	150	1-2	C_{16}–C_{18}	284.6	51.2
56	48.8	150–152	1-2	C_{16}–C_{18}	286.8	59.0
57	38.9	152	1-2	C_{16}–C_{18}	291.2	71.4
58	42.2	152	1-2	C_{16}–C_{18}	291.2	71.5
59	39.4	152	1-2	C_{16}–C_{18}	291.3	69.8
60	65.9	*	1-2	C_{16}–C_{18}	292.0	60.3
61	325.0	152–157	1-2	C_{16}–C_{18}	290.7	62.5
62	393.6†	158–162	1-2	C_{18}	295.0	65.1
63	501.0‡	Residue	1-2			

* Residue from redistillation of tenth primary fraction.
† Fractions 11 and 12 of primary distillation.
‡ Residue from primary distillation, not distilled.

rate (theory, Br 46.13 per cent). After debromination with Zn dust and alcohol, followed by saponification, subsequent acidification and extraction, 1.4 gm. of material were obtained whose analyses, as given in Table II, compared favorably with the theory for decenoic acid (mean mol. wt. 170.2, iodine number 149.3). Grün and Wirth (3) and Bosworth and Brown (4) have reported comparable values.

Tetradecenoic Acid—Fractions 18 to 30 were subjected to the same treatment as above. However, the original fraction of the dibromides obtained was still impure, as indicated by the boiling point and bromine content. The fraction was debrominated and
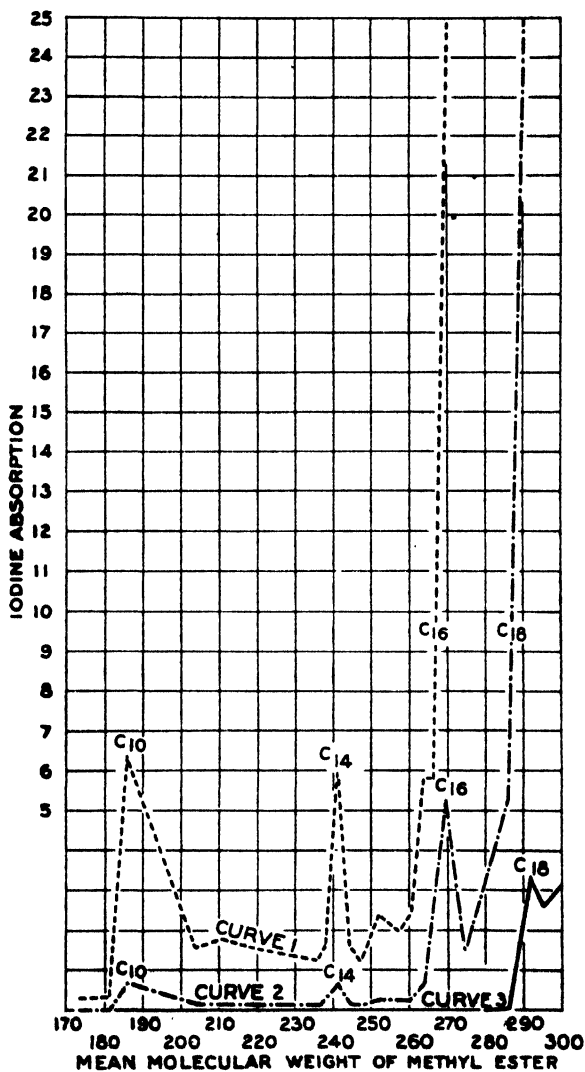
FIG. 1. Iodine absorption in relation to the mean molecular weight of the methyl ester fractions.   Curve 1, iodine absorption per fraction; Curve 2, iodine absorption per fraction + 10; Curve 3, iodine absorption per fraction + 100.

the reprepared methyl esters were fractionally distilled. Three fractions were obtained, the middle one (mean mol. wt. 239.7, iodine number 89.2) after rebromination and distillation yielded a fraction (b. p. 140–145°, 0.5 mm.) whose bromine content, as given in Table II, agreed within 1 unit per cent of the theoretical value of 39.95 per cent for methyl dibromomyristate. The values for molecular weight (225.2) and for iodine number (110.6) of the material recovered after debromination agreed well with the theoretical values for tetradecenoic acid (mol. wt. 226.3, iodine

TABLE II

Examination of Unsaturated Acids, Their Methyl Esters, and Bromine Addition Products Obtained from Fractions 4 to 48

Compound	B.p.	Pressure	Mean mol wt.	Bromine	Refractive index at 20°	I No.
	°C.	mm		per cent		
Methyl dibromocaprate	120–123	0.5		44.02	1.4925	
Decenoic acid	155–158	14.0	173.0		1.4468	147.9
Methyl dibromomyristate	140–145	0.5		38.96	1.4890	
Myristoleic acid (tetradecenoic)	183–186	14.0	225.2		1.4558	110.6
Methyl dibromopalmitate	165–170	0.5		37.24	1.4857	
Methyl palmitoleate	110–111.5	0.5	268.1		1.4498	94.0
Palmitoleic acid (hexadecenoic)	Obtained from ester by saponification		253.5		1.4587	99.4

number 112.2). Bosworth and Brown (4) reported values for the dibromide which are in good agreement with the values reported here.

Palmitoleic Acid—As already indicated, no data have been reported on the presence of palmitoleic acid in the milk fat of the cow or goat. However, the distillation data suggested its presence and confirmatory work was accordingly undertaken.

Fractions 34 to 48, when combined and subjected to the lead salt-alcohol separation, yielded a "liquid" acid fraction with a mean

molecular weight of 261.9 and iodine number of 69.5. The methyl esters of this fraction were fractionally distilled into two portions: Fraction A, boiling range of 110–122° at 0.5 to 1.0 mm. pressure and Fraction B, boiling range 122–135° at 0.5 to 1.0 mm. pressure. Fraction A was brominated in anhydrous ether at 0° and after removal of the ether the residue was fractionally distilled. A fraction of 14.0 gm., the bulk of which distilled sharply at 170° at 0.5 mm. pressure, proved to be nearly pure methyl dibromopalmitate. This bromide was a heavy amber-colored liquid, $n_D^{20} = 1.4587$, and contained 37.24 per cent bromine as compared to the theoretical percentage of 37.35. After determination, the reesterified recovered material was distilled, yielding 8.5 gm. of nearly pure methyl palmitoleate, as indicated by the data given in Table II.

The acid obtained from the ester fraction also gave values (Table II) in good agreement with theory. Upon hydrogenation of a portion of the acid with Pd-BaSO$_4$ used as catalyst, the crystallized product gave the accepted value of 62.5° for the melting point of palmitic acid. The data establish without doubt the presence of palmitoleic acid in goat milk fat.

Oleic Acid—The presence of some oleic acid accompanying the palmitoleic acid in the fractions reported above was demonstrated by further examination of Fraction B with a boiling point of 122–135° at 0.5 to 1.0 mm. pressure. Upon redistillation, a fraction of 5.1 gm. was obtained, which had a mean molecular weight of 287.5 and an iodine number of 89.2. A portion of the acids obtained from this fraction, after hydrogenation with Pd-BaSO$_4$ as catalyst, yielded a saturated product which after several crystallizations gave the following analyses: molecular weight 284.2, melting point 68.9. Stearic acid, the hydrogenation product of oleic acid, has a molecular weight of 284.3 and a melting point of 69.3°. The presence of oleic acid was also established in later fractions, incidental to a search for higher unsaturated acids.

Linoleic Acid—Since the boiling points of methyl oleate and methyl linoleate are nearly identical, the fractions containing the bulk of the methyl oleate should also contain methyl linoleate if that ester were present. Accordingly, Fractions 57 to 62 were subjected to lead salt-ether separations. The mean molecular weights of the liquid acids from Fractions 57 to 61 ranged from

281.6 to 283.3 and the iodine numbers from 88.8 to 91.8; the liquid acids of Fraction 62 had a mean molecular weight of 284.2 and an iodine number of 99.1.   These unsaturated fractions were brominated in anhydrous ether at 0°.   Traces of ether-insoluble bromides were obtained from Fraction 61 and a larger amount (0.264 gm.) from Fraction 62.   The weights of the samples taken for lead salt separations range from 6.4 to 7.0 gm.   These insoluble bromides proved to be identical and had an average bromine content of 67.6 per cent, which corresponds closely with the theoretical amount of 67.8 per cent for octabromoarachidic acid. The bromides blackened at 230–232° without showing a definite melting point.

The ether was removed from each of the above brominations by distillation and low boiling petroleum ether added.   The bromides dissolved completely and the solutions were stored in the refrigerator at 0–5°.   After several days storage no crystals of tetrabromide were obtained.   A slight precipitate was obtained from Fraction 62, which proved to be octabromides.

While traces of linoleic acid may possibly have been present in the sample investigated, the data agree with that of Bosworth and Brown on cow's milk fat in support of the conclusion that this acid was not present in appreciable amounts.

Highly Unsaturated Acids in Fraction 63—Fraction 63, the undistilled residue from the primary distillation, had a dark red coloration.   Determination by the modified Kerr-Sorber method (14) showed the presence of 0.13 per cent of unsaponifiable material as a residual product from the entire sample of butter fat.

The fatty acids of Fraction 63 were subjected to the lead salt-alcohol separation and the liquid acids converted to their methyl esters and fractionally distilled.   The fractions obtained upon bromination yielded both ether-insoluble and ether-soluble bromides.   The results given in Table III show a very close agreement between the average bromine content of the ether-insoluble bromides (66.7 per cent) and the theory for methyl octabromoarachidate (66.8 per cent).   The melting point, indistinct though it was with blackening at 228°, is in agreement with the observations reported.   Undoubtedly arachidonic acid was present in the sample.

The small amount of bromides (0.60 gm.) soluble in ether but

insoluble in petroleum ether represents all that was obtained from the entire sample. The bromine content (64.2 per cent) agrees with the theory for the methyl ester of a C_{22} acid octabromide (64.9 per cent) more closely than for methyl octabromoarachidate. This bromide was a pale orange, soft, amorphous, light powder, whereas methyl octabromoarachidate is a hard, nearly white, somewhat granular material. Bosworth and Sisson (15) reported evidence indicating that the octabromoarachidate as usually obtained (m. p. 228–230°) is a mixture of at least two isomers. They obtained fractions of these bromides melting at 162°, 208°, and 262° from butter fat from cow's milk. No evidence of the presence of linoleic acid was found in Fraction 63.

TABLE III

Data Obtained from Distillation of Methyl Esters of Liquid Acids of Fraction 63

Fraction No.	Weight	B.p. at 1 mm.	Mean mol. wt.	I No.	Ether-insoluble bromides			Ether-soluble, petroleum ether-insoluble bromides		
					Weight	M.p.	Bromine	Weight	M.p.	Bromine
	gm.	°C.			gm.	°C.	per cent	gm.	°C.	per cent
63-a	149.1	132–135	292.5	89.5	0.40	228	66.3	None		
63-b	121.6	135–145	300.0	92.7	0.87	228	67.0	"		
63-c	58.2	145–180	316.0	105.5	2.96	228	66.7	0.60	148–150	64.2

Saturated Acids from Butyric to Stearic Acid—Little difficulty was encountered in isolating in pure form the acids of even number of C atoms from caproic to stearic from those fractions whose molecular weights approached closely the theoretical value for their esters. The accepted values for the melting points of caprylic, capric, myristic, palmitic, and stearic acids were obtained after a single crystallization. However, it was necessary to redistil the fractions containing methyl laurate in order to obtain the acid of accepted melting point 43.6°.

Butyric acid was isolated from the water extract of the total mixed acids. Caproic acid, obtained from Fractions 1, 2, and 3, boiled at 200–202°.

Saturated Acids of Higher Molecular Weight Than Stearic Acid—The analyses of the saturated acids obtained from the lead salt-

alcohol separation of the fatty acids of Fraction 63, following a crystallization from alcohol, gave the first indication of the presence of saturated acids of high molecular weight. A total of 159.7 gm. of solid acids with a mean molecular weight of 290.2 and an iodine number of 1.6 was obtained.

The methyl esters of these acids were fractionally distilled from a Claisen flask, modified as previously described. The results of the distillation and mean molecular weights of the fractions are

TABLE IV

Distillation of Methyl Esters of Solid Fatty Acid Obtained from Lead Salt-Alcohol Separation of Fraction 63

Fraction No.	B.p. at 1 mm.	Weight	Mean mol. wt. of esters	Crystallization of acids	
				Mean mol. wt.	M.p.
	°C.	gm.			°C.
63-a	135–142	27.1	297.0 ⎫		
63-b	142	30.5	299.2 ⎪		
63-c			⎪	284.5	69.3
63-c	142	36.9	297.0 ⎬		
63-d	142	33.0	298.3 ⎪		
63-e	142–144	25.7	298.6 ⎭		
63-f	144–150	7.2*	303.6	286.5	68.0–68.5
63-g	150–155	2.1	319.2	321.0	66.8–67.0
63-h	Residue	4.7	377.6	378.9	74.1–74.3
63-h-a†	170–180	3.6	381.2	368.7	74.5
63-h-b	Residue	0.13	412.8		78.0

* 5.1 gm. of impure stearic acid (mean mol. wt. 286.5, m.p. 68.0–68.5°) were obtained from this fraction as first crop; 1.9 gm. of crystals (mean mol. wt. 310.2, m.p. 66.0–66.5°) were obtained from the mother liquors.

† The acids from Fraction 63-h after crystallization (mol. wt. 378.9) were esterified and distilled to give Fractions 63-h-a and 63-h-b.

given in Table IV. Fractions 63-a, 63-b, 63-c, 63-d, and 63-e, because of their similar boiling points and molecular weights, were combined. The acids obtained from these fractions after one crystallization had a mean molecular weight of 284.5 and a melting point of 69.3°, which indicate nearly pure stearic acid. Fraction 63-f (Table IV) upon crystallization yielded 1.9 gm. of acids with a mean molecular weight of 310.2 and 5.1 gm. of impure stearic acid, which had a mean molecular weight of 286.5 and melted at 68.0–68.5°.

Because Fraction 63-g was comparatively small and the preceding fraction was largely stearic acid, it was considered doubtful if any C_{20} or C_{22} saturated acids were present in more than traces. This view was also supported by the abrupt increase in molecular weight of Fraction 63-h and also by the low melting point of the higher molecular weight fraction obtained from Fractions 63-f and 63-g.

The mean molecular weights of the acids obtained from Fraction 63-h indicated a mixture of C_{24} and a higher acid, probably cerotic acid. The results of the analyses of Fractions 63-h-a and 63-h-b confirmed this view. The results of these analyses on Fraction 63 indicate that the saturated acid consisted principally of stearic acid, along with small amounts of lignoceric acid ($C_{24}H_{48}O_2$) and traces of cerotic acid ($C_{26}H_{52}O_2$). The presence of arachidic acid ($C_{20}H_{40}O_2$) or behenic acid ($C_{22}H_{44}O_2$) is questionable.

Estimation of Component Fatty Acids—In distillations of mixed saturated and unsaturated esters, it has been observed that the unsaturated acids are distributed chiefly with the fractions containing saturated acids of 2 less carbon atoms and of the same number of carbon atoms.

In the present work the assumption has been made that the unsaturation of Fractions 1 to 18 was due to methyl decenoate, Fractions 19 to 28 to methyl tetradecenoate, Fractions 29 to 49 to methyl palmitoleate, Fractions 50 to 59 to methyl oleate, and Fractions 60 to 63 to methyl oleate and methyl arachidonate. This assumption was, in general, supported by the findings reported. The calculations of the unsaturated components of Fractions 60 to 62 were based on the iodine numbers of the unsaturated acids as obtained by the lead salt-ether separations. The calculations of the unsaturated acids of Fraction 63 were based on the results reported in Table III.

After the amounts of unsaturated esters in a given fraction were calculated, the saponification number of the saturated esters in that fraction was calculated, from which the saturated components were deduced on the assumption that not more than two are present.

The results of the calculations are given in Table V. Since decenoic and tetradecenoic acids are present in very small amounts, the assumption made for their calculation should not seriously affect the other components.

Results of the calculations which are given in Table V show excellent agreement with those obtained by Dhingra (8) in his work on milk fat from Indian goats with the method developed by Hilditch and coworkers (5, 6) for most of those acids present in considerable amounts. However, it should be recalled that he does not report decenoic, tetradecenoic, palmitoleic, and arachidonic acids and does report some linoleic acid, whereas its presence

TABLE V

Component Fatty Acids in Entire Sample of Goat Milk Fat

Saturated				Unsaturated		
Acid	Weight*	Per cent by weight	Acid series	Acid	Weight*	Per cent by weight
	gm.				*gm.*	
Butyric............	69.3	2.1	C_4			
Caproic...........	63.8	1.9	C_6			
Caprylic...........	88.3	2.7	C_8			
Capric.............	261.0	7.9	C_{10}	Decenoic..........	5.4	0.2
Lauric.............	114.8	3.5	C_{12}			
Myristic...........	336.0	10.2	C_{14}	Tetradecenoic	12.9	0.4
				Hexadecenoic (pal-		
Palmitic...........	943.0	28.7	C_{16}	mitoleic)........	68.2	2.1
Stearic............	265.2	8.1	C_{18}	Oleic.............	1028.0	31.2
Saturated acid of				Arachidonic‡ and		
higher mol. wt.				(C_{22}?)...........	23.0	0.7
than stearic†.....	14.6	0.4	C_{18}			

* Corrected to complete recovery.

† Calculated as tetracosanoic (lignoceric), since the examination of Fraction 63 indicated that this acid is present in greater amount than arachidic or cerotic acid.

‡ Calculated as arachidonic acid although evidence of a C_{22} acid containing four double bonds was found.

was not detected in the present work. Dhingra also reported arachidic acid present, whereas the evidence found in this investigation indicates lignoceric rather than arachidic acid.

SUMMARY

A composite sample of fat from goat milk, consisting of 3.5 kilos, was converted to methyl esters, and fractionated into 63 fractions for determination of the component fatty acids.

A study of the distillation data indicated the presence of decenoic, tetradecenoic, palmitoleic, oleic, caproic, caprylic, capric, myristic, palmitic, and stearic acids. Subsequent examination of the fractions established the presence of these acids and also of arachidonic acid.

Traces of an octabromide of an unknown acid were found (probably a C_{22} acid or an impure isomer of arachidonic acid).

A mixture of saturated acids with higher molecular weight than stearic acid was isolated, consisting principally of tetracosanoic acid along with traces of cerotic acid.

Estimations on the content of the component fatty acids present show general agreement with previously published analyses except for the absence of linoleic acid and the presence of small percentages of decenoic, tetradecenoic, and hexadecenoic acids in the sample.

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A METHOD TO DETERMINE SMALL AMOUNTS OF CITRIC ACID IN BIOLOGICAL MATERIAL*

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Citric acid has long been known to play a part in the general organic acid metabolism of both plants and animals. The detailed study of citric acid metabolism has, however, been seriously circumscribed by the lack of accurate methods to determine this substance in the small quantities generally available in actual physiological work. Of the several methods (1–7) that have been proposed only that of Thunberg lays any claim to accuracy in dealing with quantities of citric acid less than 1 mg., and most of the methods require much larger amounts.

The Thunberg method has been quite widely applied in biological work (8–11). It employs the citric acid dehydrogenase of cucumber seed extracts, and involves observations of the time necessary for the decolorization of methylene blue. The technique presents many difficulties, and the calculations may be carried out in different ways with widely varying results; the method is accordingly far from satisfactory.

In the course of the development of their method for the determination of citric acid in plant tissues, Pucher, Vickery, and Leavenworth (5) noted that a yellow to red color is formed in the aqueous phase when pentabromoacetone in petroleum ether solu-

tion is treated with a solution of sodium sulfide. A few observations showed that this color is proportional in intensity to the quantity of pentabromoacetone in the solution, and the possibility was clearly evident that a colorimetric method suitable for the determination of small amounts of citric acid might be found. The present paper describes such a method.

The procedure, in brief, consists in the oxidation of from 0.1 to 1.0 mg. of citric acid by potassium permanganate in the presence of bromine. The pentabromoacetone produced is extracted from the oxidation mixture with petroleum ether and treated with aqueous sodium sulfide. The colored substance that forms in the aqueous phase is stabilized by the addition of pyridine, and the intensity of the color is determined in a Pulfrich spectrophotometer. The quantity of citric acid originally present is then derived from the calibration curve of the instrument.

Reagents

Sulfuric acid, 50 per cent. A mixture of equal volumes of concentrated acid and water.

Citric acid, stock solution, 1 cc. equivalent to 10 mg. of anhydrous citric acid. The purity of a sample of commercial citric acid is established by titration and the stock solution is then prepared with 1.0 N sulfuric acid as solvent.

Bromine water. Saturated aqueous solution.

Potassium bromide, 1.0 M. 11.9 gm. diluted to 100 cc.

Potassium permanganate, 1.5 N. 47.4 gm. diluted to 1000 cc.

Sodium sulfide, 4 per cent. 4 gm. of crystalline sodium sulfide diluted to 100 cc., prepared fresh every 2 to 3 days. Centrifuged or filtered before use.

Ferrous sulfate. 20 gm. of crystalline ferrous sulfate and 1 cc. of concentrated sulfuric acid diluted to 100 cc.

Petroleum ether. Boiling point 35–50°. Occasional lots of petroleum ether have been found unsatisfactory. It must therefore be tested with known amounts of citric acid before use.

Pyridine. Redistilled from Eastman's Practical grade; boiling point 112–117°.

Pyridine, 50 per cent. A mixture of equal volumes of redistilled pyridine and water.

Hydrogen peroxide. 3 per cent solution.

Trichloroacetic acid.   10 per cent solution.

Preliminary Treatment and Oxidation of Citric Acid—Of the solution to be analyzed an aliquot part that contains not more than 1.0 mg. of citric acid is transferred to a 150 cc. beaker, together with water to make a volume of approximately 75 cc.; 3 cc. of 50 per cent sulfuric acid and a few angular quartz pebbles are added, and the solution is boiled for about 10 minutes. This step is essential in order to decompose unknown substances which subsequently combine with bromine to form ether-soluble products. The solution, which should now have a volume of approximately 40 cc., is cooled to room temperature, preferably in an ice bath, an excess of bromine water (usually 3 cc.) is added, and the mixture is allowed to stand 10 minutes. If a precipitate forms, the solution is allowed to stand 20 minutes more, bromine water being added from time to time as necessary to insure an excess. The solution is then transferred to a 50 cc. centrifuge tube, the precipitate is centrifuged down, and the supernatant fluid is poured into a 125 cc. pear-shaped separatory funnel; rinsing of the tube and recentrifugation of the precipitate are unnecessary in routine analyses if the tube is perfectly clean. 2 cc. of potassium bromide and 10 cc. of potassium permanganate are then added. After being allowed to stand for 10 minutes, the solution is decolorized by the addition of the requisite amount of ferrous sulfate solution.

Extraction of Pentabromoacetone—The mixture is shaken with 25 cc. of petroleum ether, the aqueous layer is drawn off, and the ether is washed once with 5 to 10 cc. of water, the wash fluid being added to the aqueous solution. The ether is then transferred to a second pear-shaped funnel, and the aqueous solution is returned to the first and reextracted as before, the second ether extract being added to the first. The combined extracts are then washed four times with 5 cc. portions of water.

Photometric Determination of the Pentabromoacetone—The washed petroleum ether is shaken successively with 3, 2, and 1 cc. portions of filtered sodium sulfide solution, these being quantitatively drawn off into a 10 cc. volumetric flask containing 3.5 cc. of pyridine. The solution is made to volume with 50 per cent pyridine and, by means of a Pulfrich spectrophotometer, the extinction coefficient is determined within 30 minutes in a cell of appropriate

length, with a light filter No. S-43.   Water is used in the control cell of the instrument.   The amount of citric acid present in the original solution is then derived from the calibration curve.

The calibration curve is prepared by analyzing a series of solutions that contain from 0.1 to 1.0 mg. of citric acid, the preliminary boiling and treatment with bromine being omitted.

These solutions are secured by suitable dilution of the citric acid stock solution.   Table I shows the values obtained in this laboratory.   When plotted, the data give a straight line which, however, does not pass through the origin, probably owing to the presence of traces of iron.   This may be inferred from the fact

TABLE I

Calibration Data to Determine Citric Acid in the Pulfrich Spectrophotometer

Light filter No. S-43; cell length, 1 cm.

Decolorizing agent	Citric acid	No. of determinations	Extinction coefficient
	mg.		
Ferrous sulfate	0.10	5	0.155 ± 0.007
	0.30	7	0.361 ± 0.011
	0.50	6	0.581 ± 0.014
	1.00	4	1.119 ± 0.003
3% hydrogen peroxide	0.10	5	0.110 ± 0.006
	0.20	5	0.217 ± 0.010
	0.25	6	0.270 ± 0.014
	0.30	4	0.328 ± 0.010
	0.50	3	0.540 ± 0.015
	1.00	5	$1.08 \ \pm 0.025$

that if hydrogen peroxide is used as a decolorizing agent, a slightly different calibration curve is obtained which does pass through the origin.   Data for this curve are given in the lower part of Table I.

Preparation of Biological Material for Citric Acid Analysis

Urine—Portions of from 5 to 10 cc. of dog urine or of 0.2 to 1.0 cc. of human urine usually contain suitable quantities of citric acid for analysis.

Blood—1 volume of whole blood or of plasma is added to 4 to 9 volumes of 10 per cent trichloroacetic acid, and the mixture is stirred and allowed to stand 10 minutes before being filtered or centrifuged.   The aliquots removed should contain about 0.1

mg. of citric acid; that is, 10 cc. or less of whole blood. It is important that the blood be added to the trichloroacetic acid immediately after being drawn from the animal; a loss of as much as one-fifth of the citric acid contained may occur within 1 hour at 37° or 2 hours at room temperature.

Feces—The fecal matter is ground with water which has been acidified to Congo red with sulfuric acid, and an aliquot portion is mixed with an equal volume of 10 per cent trichloroacetic acid. After filtration, an aliquot that represents one-fifth to one-tenth of 1 day's collection is taken for analysis.

Animal Tissue—Organ or muscle tissue is ground with sand in a mortar with several portions of 10 per cent trichloroacetic acid. An aliquot part of the filtrate that represents 10 gm. of the original tissue is usually suitable for the analysis.

TABLE II

Citric Acid Determinations in Dog Urine

Range of citric acid content	No. of determinations	Average deviation from mean of duplicates
mg. per 100 cc.		*per cent*
<0.10	14	9.0
0.10–0.20	100	2.6
0.20–0.30	58	1.8
0.30–0.50	36	2.1
0.50–1.00	34	4.6

Plant Tissue—Although it is sometimes feasible to determine citric acid directly in water extracts of plant tissues, the possibility of the presence of interfering substances makes this procedure inadvisable. Preparation of the "organic acid fraction" by ether extraction according to the method outlined by Pucher, Vickery, and Wakeman (12) is much to be preferred and, furthermore, permits the determination of the total organic acidity and the malic acid. It should be noted that, in analyzing plant tissues, decolorization of the oxidation mixture with hydrogen peroxide instead of with ferrous sulfate is essential; this procedure has been described by Pucher, Vickery, and Wakeman (13).

Experimental Applications

Table II shows in summary form the results of all the determinations made on dog urine in a single month. The last column gives

the average deviation of the individual determinations from the respective means of duplicates, and serves to show the degree of reproducibility that may be attained. It is clear that, with quantities between 0.1 and 0.5 mg., the deviation is less than 3 per cent and in general, save with extremely small amounts, is less than 5 per cent. In 88 analyses of dog blood, for which the data are not shown, the average deviation was 3 per cent.

Table III shows the accuracy with which citric acid added to the urine and blood of dogs may be recovered. The recovery from dog urine is unusually low, probably owing to adsorption of a little

TABLE III

Recovery of Citric Acid Added to Urine and Blood of Dogs

	Original sample	Added	Total present	Total found	Recovery
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Urine	0.210	0.300	0.510	0.485	92
				0.470	87
				0.485	92
				0.485	92
				0.490	93
	0.720	0.200	0.920	0.900	90
				0.910	95
				0.910	95
Blood	0.213	0.237	0.450	0.450	100
				0.460	104
	0.120	0.120	0.240	0.240	100
				0.240	100
				0.250	108

citric acid on the voluminous precipitate that appears when this urine is treated with bromine. A similar phenomenon does not usually occur with human urine, and recoveries of 99 per cent were readily secured. The recoveries from dog blood, which likewise gives no appreciable precipitate with bromine, were satisfactory. It is clear that, although the relative accuracy of the method is in general better than 5 per cent, the absolute magnitude of the results in certain specific cases may be somewhat low, owing to the loss of citric acid by adsorption during the preparation of the solution for analysis.

Specificity of the Analytical Method—The following substances in

100 to 200 mg. quantities gave no coloration under the conditions described for the estimation of citric acid: acetone, glycogen, acid hematin, creatine, creatinine, cholesterol, amytal, urea, taurine, toluene, acid digest of casein, allantoin, and uric, hippuric, lactic, malic, oxalic, tartaric, succinic, maleic, and fumaric acids. β-

TABLE IV

Citric Acid Content of Tissues and Excreta

	Citric acid
	mg. per 100 cc.
Whole blood, dogs, 20 hrs. postabsorptive	0.9 – 1.9
Saliva, human	0.04– 1.3
	mg. per 24 hrs.
Urine, 6 dogs on low citric acid diet	1.3 –50
Feces	
Dog 3. Normal diet	0.4 – 0.8
48 hr. period following ingestion of 10 gm. sodium bicarbonate	0.8
24 hr. period following ingestion of 14.1 gm. sodium citrate	1.9
48 hr. period following ingestion of 14.1 gm. sodium citrate	3.8
Dog 4. Normal diet	0.7
72 hr. period following ingestion of 8 gm. citric acid	0.8
96 hr. period following ingestion of 16 gm. citric acid	1.4
Tissues	*mg. per 100 gm.*
Dog 7.* Kidney	1.2
" 7. Muscle, abdominal	1.1
" 7. Liver	0.1
" 8.* Kidney	1.9
" 8. Muscle, heart	1.5
" 8. Liver	0.6

* Dog 7 suffered from malnutrition and from a bone infection. Dog 8 suffered from cancer. The tissues selected for analysis presented no gross abnormalities.

Hydroxybutyric acid and the ethyl ester of acetonedicarboxylic acid in quantities of 100 mg. each gave colors equivalent to from 0.1 to 0.5 and 0.9 mg. of citric acid respectively. The latter compound is more stable than the free acid, which would probably be decomposed to acetone during the preparation of the solution for analysis (14) and so eliminated as a source of error in the deter-

mination of citric acid. These observations indicate that, although the colorimetric procedure is specific for citric acid in the presence of certain commonly occurring constituents of biological mixtures, compounds such as non-volatile ketones and keto acids may interfere if present in abnormally large amounts. Qualitative tests

TABLE V

Citric Acid Distribution between Cells and Plasma in Dog Blood

Cell volume	Citric acid			Ratio Plasma Cells
	Whole blood	Plasma	Cells (calculated)	
per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
45	1.5	1.8	1.13	1.64
45	0.92	1.12	0.68	1.65
47	1.3	1.6	0.96	1.67
48	0.64	0.77	0.50	1.54

TABLE VI

Recovery of Citric Acid Added to Plant Tissue

2 gm. quantities of tissue were used.

	Citric acid present	Citric acid added	Citric acid found	Recovery of added acid
	mg.	*mg.*	*mg.*	*per cent*
Peat moss	0.00	0.130	0.136	105
			0.130	100
			0.140	108
			0.128	98.5
			0.130	100
	0.00	0.065	0.070	108
			0.063	97
Tobacco stem	0.154	0.256	0.412	101
			0.410	100
	0.154	0.100	0.250	96
			0.246	92
			0.255	101

for the presence of ketone bodies should always be performed on urine samples before the determination of citric acid is attempted.

Citric Acid Content of Animal Tissues and Excreta—The citric acid content of a number of animal tissues and excreta is given in Table IV to illustrate the order of magnitude of the quantities

found under certain conditions.   A more detailed discussion of citric acid metabolism will form the subject of a subsequent paper; for the present it will suffice to draw attention to the small amount of citric acid excreted by the dog.   By careful selection of foods, the citric acid intake could be reduced to as little as 20 to 45 mg. per day; on such a diet, dogs excreted from 1.3 to 50 mg. per day.

The analyses of human saliva are of particular interest, inasmuch as Leake (15) and Kuyper and Mattill (16) were unable to detect citric acid in their samples.   The data shown represent analyses of seven samples obtained by mechanical stimulation or by natural flow from six normal individuals.   In three cases, the amounts

TABLE VII

Citric Acid Content of Tobacco Plant Tissue

2 gm. quantities were used.   Aliquots that contained approximately 0.1 mg. were taken for analysis.

	Citric acid
	per cent
Stem A	0.025
	0.020
" F	0.135
	0.135
" K	0.178
	0.182
Blossom H	0.623
	0.649
Leaf A	0.83
	0.87

found in 20 to 25 cc. of saliva were of doubtful significance, but in four other cases appreciable amounts were present (0.04 to 0.33 mg. per 100 cc.).   The exceptional figure of 1.3 mg. per 100 cc. was obtained 3 hours after the subject had eaten grapefruit.   The saliva of this same individual had contained only traces of citric acid at a previous test.   The citric acid content of human saliva may therefore vary quite widely under certain conditions.

Table V gives data on the distribution of citric acid between plasma and cells in dog blood.   The values for plasma and whole blood are the averages of triplicate determinations on each sample; the values for cells are calculated.   It is clear that the cells of dog

blood contain very appreciable amounts of citric acid; this is in contrast to the findings of Nordbö and Scherstén (17) who found little or no citric acid in human erythrocytes.

In the experiments on plant tissues shown in Table VI, the citric acid was added in solution to 2 gm. portions of the dry tissue, and the mixture was treated with the requisite amount of dilute sulfuric acid and extracted with ether as described by Pucher, Vickery, and Wakeman (12). The determinations were made on the "organic acid fractions" so obtained. The recoveries are satisfactory. Table VII gives a few additional data on the citric acid content of various plant tissues, and illustrates the precision with which duplicate results are to be obtained. The last sample mentioned, Leaf A, was found to contain 0.82 per cent of citric acid when analyzed by the method of Pucher, Vickery, and Leavenworth (5).

DISCUSSION

In carrying out the present method to determine small quantities of citric acid, it is essential to follow directions closely. Each step has been made the subject of careful study, and it has been found that gross variations may lead to erratic results. The preliminary boiling of the sample with dilute acid is necessary in order to remove volatile compounds, or to decompose substances which, after oxidation, yield bromine compounds that pass into the petroleum ether and interfere with the subsequent photometric procedure.

The preliminary treatment with bromine is especially important with urine samples; an excess must be added and time allowed for the complete precipitation of derivatives. There is little danger of destruction of citric acid by the bromine. Tests have shown that, even after a delay of 1 hour, no loss occurred, and after 72 hours the results were only from 5 to 7 per cent low.

The decolorization of the oxidation mixture with ferrous sulfate instead of with hydrogen peroxide is more convenient in routine blood and urine analyses, inasmuch as no particular attention need be paid to the temperature or to the quantity of reagent used. On the other hand, if insufficient ferrous sulfate is added, the excess of bromine may not be removed and this may give rise to high values. Moreover, the washing of the ether must be very carefully conducted in order to remove the iron. Hydrogen peroxide is much

to be preferred for the analysis of plant extracts, as the simultaneous determination of malic acid is then possible; it must, however, be added slowly to the thoroughly chilled solution, and an excess must be avoided. Furthermore, a different calibration curve for the spectrophotometer must be used. Although the yellow color produced by adding sodium sulfide to the petroleum ether solution of pentabromoacetone fades rapidly in aqueous solution, it is stable for at least 30 minutes in the presence of pyridine. This permits simultaneous work on a number of samples; at least six can be conveniently dealt with at once.

SUMMARY

Quantities of citric acid of the order 0.1 to 1.0 mg. can be determined with an accuracy of ±5 per cent by oxidation to pentabromoacetone and conversion of this substance by means of sodium sulfide to a colored material that is suitable for estimation in the Pulfrich spectrophotometer. Data are given on the application of the method to the analysis of blood, urine, feces, and of both animal and plant tissues.

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THE CITRIC ACID FORMED IN ANIMAL METABOLISM*

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Citric acid was discovered for the first time as an animal metabo-
·lite in 1888, when Soxhlet and Henkel isolated it from cow's milk,
which has since been reported to contain 1 to 4 gm. per liter
(Allen, 1931).   It has been isolated from human urine (Amberg
and Maver, 1921), in which it occurs as a regular constituent
(Amberg and McClure, 1917; Fasold, 1930; Süllmann and Schaerer,
1932; Fürth *et al.*, 1934; Kuyper and Mattill, 1933; Boothby and
Adams, 1934; Schuck, 1934).   Most of the figures which have
been reported for the normal daily excretion of citric acid by
human adults fall within the range 0.2 to 1.0 gm. which was sug-
gested by Östberg (1931).   Citric acid has also been reported in
the urine of other species, including the horse (Östberg, 1931),
cow (Östberg, 1931), guinea pig (Östberg, 1931; Fürth *et al.*, 1934),
rabbit (Langecker, 1933; Fürth *et al.*, 1934), dog (Fürth *et al.*,
1934; Boothby and Adams, 1934; Pucher *et al.*, 1936), rat (Östberg,
1931), and hog (Woods, 1927; Fürth *et al.*, 1934).

Human blood serum is said to contain 1.5 to 4.0 mg. per cent of
citric acid (Scherstén, 1931); rabbit serum, 7 to 14 mg. per cent
(Kuyper and Mattill, 1933; Lindholm, 1934); and the whole blood
of dogs, 0.9 to 1.9 mg. per cent (Pucher *et al.*, 1936).   In addition,
citrate has been found in a number of other body fluids, such as

the cerebrospinal fluid (Benni, 1931; Boothby and Adams, 1934), amniotic fluid (Nitzescu and Georgescu, 1930; Boothby and Adams, 1934), aqueous humor (Nitzescu and Georgescu, 1930; Grönvall, 1930), and, contrary to earlier reports (Leake, 1923; Kuyper and Mattill, 1933), in saliva (Pucher et al., 1936). The detection of citrate in animal tissues has been reported by Gemmill (1934), and a few quantitative data are cited by Pucher et al. (1936).

This general distribution of citrate throughout the animal organism suggests that the substance must play some part in metabolism. Östberg (1931) noted a close parallelism between the daily citric acid excretion of any given individual and the pH ot the urine—whether alterations in the latter value were induced by dietary changes, by the ingestion of acids or alkalies, or by metabolic disturbances—and suggested that, at least for man, the citrates of the urine are one of the important buffer systems, and that, during alkalosis, the citrate, which is put out in greatly increased amounts, may play a significant part in conserving the fixed acid of the body. The increased citrate excretion in alkalosis has been confirmed by many other investigators (Fasold, 1930; Magnusson, 1932; Süllmann and Schaerer, 1932; Kuyper and Mattill, 1933; Boothby and Adams, 1934; Fürth et al., 1934; Schuck, 1934; Langecker, 1933). However, Boothby and Adams (1934) and Kuyper and Mattill (1933), noting instances in which the acid-base requirements did not seem to be the factor determining the excretion of citrate, suggested that this rôle has been overemphasized, and that citrate in all probability plays some other, as yet unknown, part in normal metabolism.

Despite the frequently expressed belief that the citrate of the urine is formed within the animal organism, there has been no clear cut demonstration that this is so. Of the experiments previously reported, only those of Boothby and Adams (1934) with a fasting dog preclude the possibility of a dietary origin, and the lack of quantitative data for the concentration of citrate in body tissues has left open the question of possible stores of this material which may be drawn upon in alkalosis.

In the present study, a demonstration of the existence of this citric acid of metabolic origin has been attempted, with an effort to determine its precursor, the effect of dietary and acid-base

factors upon its formation, and the interrelationship between the blood level and the urinary excretion of citrate.

Plan of Metabolism Experiments—Male and female dogs weighing 5.5 to 22 kilos received daily a constant allowance of one of the synthetic diets described in Table I, with which the various experimental supplements were mixed. Urine was collected by catheterization (with the exception of the single male, Dog 4) over

TABLE I

Composition of Experimental Diets

	Basal Diet I	Basal Diet II	High casein	High sucrose
	per cent	per cent	per cent	per cent
Casein.....................	38.3	37.4	63.8	
Sucrose....................	27.0	26.4		63.8
Lard......................	16.9	16.5	16.5	16.5
Butter.....................	6.7	6.5	6.5	6.5
Wesson salt mixture*........	3.6	3.5	3.5	3.5
Yeast.....................		6.8	6.8	6.8
Wheat embryo..............	4.6			
Bone ash..................	2.8	2.7	2.7	2.7
Citric acid†...............	0.0077	0.0226	0.0242	0.0204

* Wesson, L. G., *Science*, **75**, 339 (1932).

† Calculated from the following citric acid contents (determined by analysis): casein 0.005 per cent, yeast 0.30 per cent, wheat embryo 0.12 per cent. Butter contained less than 2 parts per million of citric acid, sucrose and lard, no citric acid.

short time or 24 hour periods. Blood was drawn from the jugular vein.

Rats kept in metabolism cages were allowed Basal Diet II and tap water *ad libitum* and urine was collected over 48 hour periods.[1]

Human subjects ingested diets of ordinary foods, described qualitatively below, and urine was collected over 24 hour periods.

Methods of Analysis—Dog urine, blood, and feces, and rat urine were analyzed for *citric acid* by the colorimetric method described in the preceding paper (Pucher *et al.*, 1936); human urine was

[1] Urine collection was made by the technique described by Mr. Max Kriss (personal communication).

treated according to the same procedure, except that the penta-bromoacetone was determined by bromide titration (Pucher *et al.*, 1934).

The *pH* of most of the dog urines was measured electrometri-cally, with the antimony-antimony trioxide electrode (Roberts and Fenwick, 1928); the pH of human urines and of a few dog urines was determined colorimetrically (Peters and Van Slyke, 1932).

Normal Ranges—The urinary excretion of citric acid on Basal Diets I and II ranged from 1.3 to 8.0 mg. (0.1 to 0.5 mg. per kilo) per day for five dogs; that of a sixth dog varied from 8.0 to 50 mg. (0.5 to 3.0 mg. per kilo) per day (Table II). Seven human sub-

TABLE II

Urinary Citric Acid of Dogs on Basal Diets I and II

Dog No.	Weight	Sex	Citric acid excretion
	kg.		*mg. per day*
1	18	♀	8.0–50
2	16–18	♀	3.0– 8.0
3	8– 8.5	♀	1.3– 3.2
4	15–16	♂	3.0– 7.0
5*	20–22	♀	2.4– 3.2
6†	5.5	♀	2.2

* Dog 5 received submaintenance diet.

† Dog 6 was pregnant.

jects on ordinary mixed diets excreted 0.356 to 1.18 gm. (5 to 20 mg. per kilo) per day. Four rats receiving Basal Diet II *ad libitum* excreted 0.32 to 0.66 mg. (1.0 to 2.0 mg. per kilo) per day (Table VI).

The postabsorptive level of blood citric acid in dogs receiving the basal diet ranged from 0.9 to 1.9 mg. per 100 cc. of whole blood. Citric acid is present both in the plasma and in the cells (Pucher *et al.*, 1936).

Dogs excreted about 0.4 to 0.8 mg. of citric acid daily in the feces (Pucher *et al.*, 1936).

Effect of Diet—A study was made of the effect of alterations in the composition of the diet on the excretion of citrate. Dogs were fed in turn the high sucrose and high casein diets described in

Table I, in which the weight of casein plus sucrose in Basal Diet II was replaced entirely by sucrose or by casein. The citrate excretion of two dogs was distinctly higher on the high sucrose diet, whereas that of the third appeared independent of the dietary change. The greater excretion on the carbohydrate ration presumably could not be attributed to the somewhat less acid urine on that diet, since the difference was equally marked when sufficient sodium bicarbonate was given in addition to the high casein diet to adjust the urinary pH to that observed with the high sucrose ration (Table III).

TABLE III

Urinary pH, Citric Acid, and Nitrogen on High Casein and High Sucrose Diets (Averages)

Dog No.	Diet	No. of days averaged	pH	Nitrogen	Citric acid
				gm. per day	*mg. per day*
3	100 gm. high casein	7	6.1	8.50	3.2
	100 " " sucrose	8	6.5	1.30	3.2
1	200 " " "	5*	6.3	3.35	44
	200 " " casein	4*	6.1	15.6	13
	200 " " " + 3 gm. NaHCO₃	4	6.4	16.4	18
2	200 " " sucrose	5*	6.3	2.00	24
	200 " " casein	4*	6.1	14.6	9.5
	200 " " " + 3 gm. NaHCO₃	4	6.4	15.3	9.5

* 2 or 3 days preliminary period on diet excluded from the average.

The early suggestion (Vaudin, 1894) that the citric acid in milk is a peculiar oxidation product of lactose, formed by the mammary gland, has been recently reiterated with some circumstantial evidence (Kieferle, Schwaibold, and Hackmann, 1925); and Fürth *et al.* (1934) have reported an increase in urinary citric acid of a hog following the ingestion of 50 gm. of lactose. However, the addition of 50 gm. of lactose to the usual daily allowance of 150 gm. of Basal Diet I for 2 successive days failed to alter the urinary excretion of citrate by the dog.

Ingestion of a meal has been reported to cause an increased rate of excretion of citrate independent of the "alkaline tide" (Kuyper and Mattill, 1933); fasting was said to decrease the concentration

of citrate in the blood of rabbits (Kuyper and Mattill, 1933; Lindholm, 1934); whereas the ingestion of glucose failed to alter either the excretion (Kuyper and Mattill, 1933) or the blood concentration (Lennér, 1934) of citric acid.   In the present study, the rate of citrate excretion has been determined during a 4 hour morning period, a 4 hour afternoon period, and a 16 hour night period.   The dogs received the usual amount of the basal diet at the beginning of either the morning or the afternoon period.   The results are given in Table IV.   There usually occurred a rise in

TABLE IV

Effect of Ingestion of Food on Urinary pH and Rate of Excretion of Citric Acid

Dog No.	Time fed	8.40 a.m.–12.40 p.m.		12.40–4.40 p.m.		4.40 p.m.–8.40 a.m.	
		pH	Citric acid	pH	Citric acid	pH	Citric acid
			mg. per hr.		*mg. per hr.*		*mg. per hr.*
1	12.40 p.m.	6.0	0.72	6.0	1.64	5.4	0.27
	12.40 "	5.6	0.15	6.6	0.63		
	12.40 "	5.8	1.60	6.5	2.30	5.7	0.70
	12.40 "	5.7	0.26	6.6	8.4	5.4	0.88
	8.40 a.m.	5.8	6.7	5.4	1.25	5.8	0.28
	8.40 "	6.8	6.0	5.7	1.28	6.2	0.40
	8.40 "		2.0		0.58		0.36
	8.40 "	5.7	1.7	5.8	0.62	6.3	0.28
2	12.40 p.m.	5.8	0.30	6.8	0.34	6.4	0.26
	12.40 "	5.8	0.31		0.31	6.3	0.26
	12.40 "	7.2	0.14	6.3	0.27	5.8	0.17
	8.40 a.m.	5.9	0.49	5.3	0.55	6.2	0.40
	12.40 p.m.	6.8	0.31	7.0	0.75	5.9	0.50
	12.40 "	6.3	0.26	6.3	0.38	5.8	0.27
3	12.40 "		0.19		0.37		0.15

citrate elimination following the meal, although the magnitude of the response varied with the different animals; the increases with Dogs 2 and 3 seem comparable with those reported by Kuyper and Mattill (1933), whereas those with Dog 1 were occasionally very much greater.   The increase apparently does not depend upon alterations in the acid-base balance of the animal—in so far as the urinary pH is an indication—since this latter value failed to increase in one-third of the cases where there was a rise in citrate excretion.   It is not a diurnal variation, inasmuch as the rate of

TABLE V

Effect of Ingestion of Food on Blood Citric Acid

Dog No.	Citric acid		
	Fasting blood	Postprandial blood	
	mg. per cent	hrs. after feeding	mg. per cent
4	0.90	2	1.20
		4	0.95
1	1.60	1.5	2.00
		4.5	1.85
2	1.90	1	1.90
		3	2.00
		5	1.80
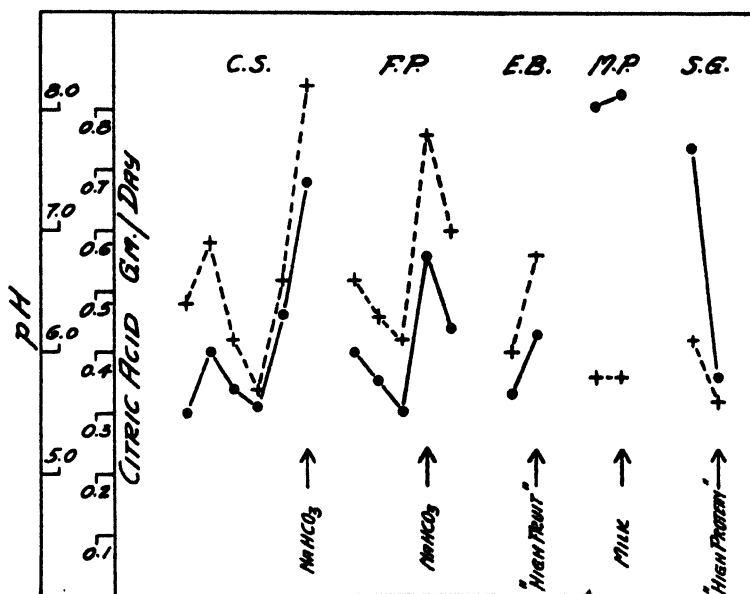
FIG. 1. The relation of citric acid excretion (solid line) to urinary pH (dash line) in human subjects. Except on the days indicated by an arrow, a freely chosen mixed diet was taken.

citrate excretion in fasting animals is practically constant throughout the 24 hour period, and since the postprandial increase occurs whether food is taken early in the morning or at noon.

In Dogs 1 and 4 there was a slight increase in the blood citrate following the ingestion of food, but in a single observation with Dog 2, no rise occurred (Table V). The very high postprandial excretion of citrate with Dog 1 is attributable to the exceptionally low renal threshold (less than 2.3 mg. per cent) noted for this animal in citric acid tolerance studies (Sherman *et al.*, 1936).

Effect of Acid-Base Factors—Fig. 1 illustrates a comparison of the daily variations in citrate excretion of human subjects with the fluctuations in urinary pH brought about (1) by chance alterations in the composition of the uncontrolled mixed diet, (2) by

TABLE VI

Effect of Alkali Ingestion on Citric Acid Excretion of Rats

Rat No.	Weight	Sex	Daily citric acid excretion	
			Basal Diet II*	Basal Diet II + 10 per cent NaHCO₃'
	gm.		*mg.*	*mg.*
1	250	♀	0.32	15
			0.46	43
2	325	♂	0.66	36
			0.52	50
3	350	♂	0.50	56
			0.40	
4†	375	♂	0.54	34
			0.48	

* Food intakes remained practically constant throughout.

† Rat 4 developed a marked hematuria on the alkali-containing diet, which disappeared when the normal diet was resumed.

ingestion of sodium bicarbonate in addition to the mixed diet, and (3) by a shift from a mixed diet to an unbalanced diet such as an exclusively milk diet, a predominantly fruit diet, and a high protein diet. These data confirm those of Östberg (1931) in demonstrating a direct parallelism between the urinary pH and the citrate excretion of any given individual. In this series, there was no evidence of a relationship between urinary nitrogen and citrate, such as that observed occasionally by Boothby and Adams (1934).

Preliminary observations of the effect of ingestion of a diet containing 10 per cent of sodium bicarbonate on the urinary excretion

of citrate were made with four rats (Table VI).   For this species also a very greatly increased elimination of citric acid was noted in alkalosis.

The close interrelationship between the daily fluctuations in the urinary pH and in the citrate excretion, noted with human subjects, was not apparent in dogs maintained on the basal diet. However, the inducement of large increases in the urinary pH by the ingestion of additional alkali was almost invariably attended by a significant rise in citrate excretion.   When the alkalosis was of only 1 to 2 days duration, this increase was relatively slight (on the average, to about 4 times the basal level); but when the ingestion of sodium bicarbonate was continued over longer periods of time, the citric acid excretion increased to 100 to 300 mg. on the 3rd or 4th day, and was maintained at this level, 20 to 60 times the basal excretion, for as long as the alkalosis was continued (more than 35 days).   Although this excretion fluctuated as much as 100 per cent from day to day, there was, with the exception of one animal, no progressive change after the first few days. The animal mentioned, in which the alkalosis produced was more severe, as measured by the urinary pH, than in the other cases, showed a gradual drop in citric acid excretion from about 300 mg. per day during the 1st week to about 100 mg. per day in the 5th week, attended by a fall in the pH toward the end of the period and by a progressive alteration in the shape of the "total organic acids" electrometric titration curve, suggestive of other adjustments of the organism to long continued alkali administration. In this series, it has not been possible to exhaust the ability of the organism to provide citrate for this increased elimination by the kidney.

These studies of prolonged alkalosis have demonstrated unmistakably that the body can continue to excrete amounts of citric acid greatly in excess of those present in the food. · For the three periods illustrated in Table VII, negative balances of 5.19, 10.31, and 7.04 gm. of citric acid, respectively, were noted.   The order of magnitude of these differences, together with the absence of demonstrable stores of preformed citrate in kidney, muscle, liver, or blood of dogs (Pucher *et al.*, 1936), supports the view that citric acid may be formed in the metabolism of the dog.

In the hope of obtaining some indication of the nature of the

precursor from which this metabolic citric acid is derived, the high casein and high sucrose diets were substituted for the basal diet given to animals receiving continuous daily doses of a constant amount of sodium bicarbonate. To study the effect of addition or removal of sucrose or casein alone, high casein-high sucrose periods were also included in which the animal received both casein and sucrose in the amounts ingested on the high casein and

TABLE VII

*Citric Acid Excretion of Dogs during Prolonged Alkalosis**

Dog No.	Diet	Days on diet	Total citric acid	
			Ingested	Excreted
			gm.	*gm.*
4	Basal I	35	0.40	5.59
2	Basal II	20	0.90	2.87
	High casein	7	0.34	0.27
	" sucrose	9	0.37	7.64
	" casein-high sucrose	5	0.24	1.10
	" casein	4	0.19	0.47
Total.............................		45	2.04	12.35
1	High casein	6	0.29	1.55
	" casein-high sucrose	6	0.29	1.17
	" sucrose	6	0.24	5.14
Total.............................		18	0.82	7.86

* Each dog received 20 gm. of sodium bicarbonate daily. Dog 4 ingested 150 gm. of diet daily. Dogs 1 and 2 ingested 200 gm. daily of basal Diet II, high casein, and high sucrose diets, respectively, and 327 gm. of high casein-high sucrose mixture (200 gm. of high casein diet plus 127 gm. of sucrose).

high sucrose diets, respectively, in addition to a constant amount of the other constituents of the diet. Fig. 2 illustrates the results obtained with two dogs. In both animals, the high sucrose diet favored the excretion of over 1 gm. per day of citric acid, very much more than had been observed under any other conditions. Addition of casein to the high sucrose diet caused a prompt drop in citrate excretion (Dog 2), whereas removal of casein from the high casein-high sucrose diet induced a marked rise (Dog 1).

Variable results attended alterations in the intake of sucrose, addition of sucrose to the high casein diet causing a significant fall (Dog 1); its removal from the high casein-high sucrose diet, also a slight decrease (Dog 2) in citric acid excretion. A possible explanation suggested by these experiments at the present time is that the metabolism of casein, because of the potentially acid and buffer materials liberated by the process, moderates the severity
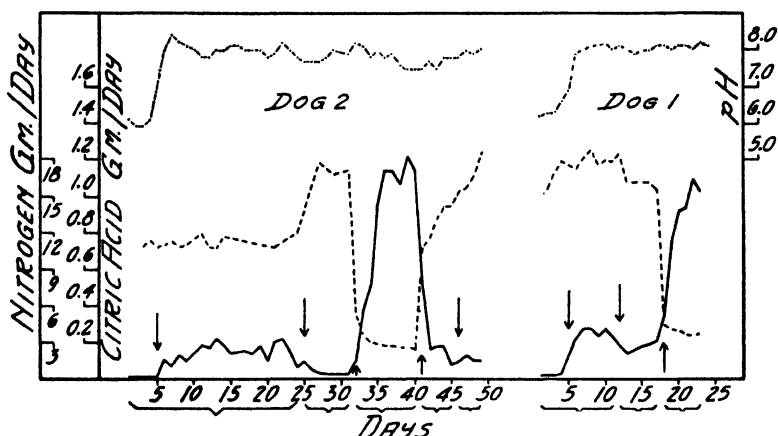
Fig. 2. The effect of alkalosis and diet on the urinary pH (dash and dot line), citric acid (solid line), and nitrogen (dash line) excretion. The daily administration of 20 gm. of sodium bicarbonate was begun on the 5th day in each series and continued throughout the experiment, with the exception of days 29 to 31, when Dog 2 received 24 gm., and days 38 to 40, when Dog 2 received 17 gm. of sodium bicarbonate. The diets were as follows: Dog 2, days 1 to 24, Basal Diet II; days 25 to 31 high casein; days 32 to 40, high sucrose; days 41 to 45, high casein-high sucrose; days 46 to 49, high casein. Dog 1, days 1 to 11, high casein; days 12 to 17, high casein-high sucrose; days 18 to 23, high sucrose.

of the alkalosis produced by a constant amount of· sodium bicarbonate, and *ipso facto* diminishes the citrate response. It should be noted, however, that the pH of the urine (by our present method of collection, which undoubtedly permits the loss of much carbon dioxide) failed to reflect such postulated variations in the acid-base condition of the organism.

These experiments fail to point clearly to either protein or carbohydrate as the precursor of the metabolic citric acid, inasmuch

as neither increased amounts of casein nor increased amounts of
sucrose *per se* induced a rise in citrate excretion.   On the other
hand, neither can be eliminated conclusively, since, even on the
sucrose-free high casein diet, there was available to the organism
considerable glucose derivable from the protein of the diet; where-
as, on the casein-free high sucrose diet, protein was being metab-
olized to the extent represented by 1.6 to 3.6 gm. per day of urinary

TABLE VIII

*Effect of Equivalent Amounts of Sodium Bicarbonate and Sodium Acetate on
Urinary pH and Citrate Excretion*

Dog No.	Sodium bicarbonate		Sodium acetate	
	pH	Citric acid	pH	Citric acid
		mg. per day		*mg. per day*
2*	(6.0)	(4.0)	(5.8)	(4.5)
	8.3	7.0	8.2	9.0
	8.4	6.0	8.4	6.0
	(6.9)	(4.0)	(6.6)	(5.0)
3†	(6.7)	(3.8)	(6.3)	(5.5)
	8.0	25	8.0	35
	8.0	72	8.0	27
	(7.0)	(29)	(6.6)	(5.5)
	(6.7)	(7.5)	(6.2)	(2.0)

Data in parentheses refer to control days preceding and following those
on which alkali was given.

* Dog 2 received 20.0 gm. of $NaHCO_3$ and 32.4 gm. of $NaC_2H_3O_2 \cdot 3H_2O$,
respectively, administered by stomach tube in two doses daily, for 2 suc-
cessive days.

† Dog 3 received 10.0 gm. of $NaHCO_3$ and 16.1 gm. of $NaC_2H_3O_2 \cdot 3H_2O$,
respectively, administered by stomach tube in two doses daily, for 2 suc-
cessive days.

nitrogen, about 1 gm. of which was of dietary origin.   However,
it seems rather unlikely that the metabolic citric acid arises exclu-
sively from protein, since the highest values for the citrate excre-
tion on the casein-free diet corresponded to the lowest values for
the urinary nitrogen.   This is apparent in Fig. 2, and is most
strikingly illustrated by a day (not shown in Fig. 2) on which
Dog 2 excreted 1.64 gm. of citric acid and only 1.65 gm. of urinary
nitrogen.

The intended extension of this series of experiments to include

the effect of alterations in the dietary fat content unfortunately could not be completed at the present time.

The suggestion was made by Fürth *et al.* (1934) that citrate is derived from carbohydrate through acetate, a view based upon the belief (recently discredited by Butkewitsch *et al.*, 1934) that

TABLE IX

Effect of Equivalent Amounts of Sodium Bicarbonate; Sodium Citrate, and Citric Acid on Urinary pH and Citrate Excretion

Dog No.	Sodium bicarbonate		Sodium citrate		Citric acid	
	pH	Citric acid	pH	Citric acid	pH	Citric acid
		mg. per day		*mg. per day*		*mg. per day*
2*	(5.9)	(8.5)	(5.9)	(7.5)	(5.7)	(6.0)
	7.8	6.0	8.2	20	5.7	8.0
	8.3	10.0	8.5	21	5.7	7.0
	(6.5)	(3.5)	(6.7)	(6.0)	(5.8)	(5.5)
	(6.0)	(4.0)	(6.0)	(6.5)		
	8.3	7.0	7.9	10		
	8.4	6.0	8.3	14		
	(6.9)	(4.0)	(6.4)	(4.0)		
3†	(6.4)	(3 2)	(6.2)	(1.5)	(6.2)	(3.3)
	8.3	4.0	7.9	33	6.3	4.6
	(6.7)	(3.3)	(6.4)	(4.0)	(6.2)	(3.9)
	(6.0)	(1.7)	(6.0)	(2.2)		
	(6.3)	(2.8)	(6.3)	(2.9)		
	8.1	3.4	8.1	35		
	(6.3)	(2.3)	(7.0)	(8.1)		
	(6.3)	(2.9)		(4.6)		

Data in parentheses refer to control days preceding and following those on which alkali was given.

* Dog 2 received 20.0 gm. of $NaHCO_3$, 28.6 gm. of $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$, and 16.8 gm. of $C_6H_8O_7 \cdot H_2O$ per day, respectively, administered by stomach tube in two doses daily, for 2 successive days.

† Dog 3 received 10.0 gm. of $NaHCO_3$, 14.3 gm. of $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$, and·8.4 gm. of $C_6H_8O_7 \cdot H_2O$ per day, respectively, administered in a single dose, for 1 day only.

certain molds accomplish the conversion, acetic acid → citric acid. In support of this view, Fürth *et al.* report the observation that, in the hog, sodium acetate was invariably somewhat more effective in stimulating citrate excretion than the equivalent amount of sodium as bicarbonate.   In the present study, similar experiments

with dogs showed no such favorable effect of the acetate radical, supplements of sodium acetate producing an increase in citrate excretion no greater than that caused by the equivalent amount of sodium bicarbonate under identical conditions (Table VIII).

It seemed possible that the increased excretion of citrate during alkalosis might be due to an impairment of the oxidative capacity of the organism for citrate, analogous to the decreased glucose tolerance which was observed by Haldane (1924) in alkalosis. To test this possibility, dogs were given, successively, chemically equivalent amounts of sodium bicarbonate, sodium citrate, and citric acid (Table IX). The citric acid excretion following the ingestion of sodium citrate exceeded in every case that following

TABLE X

Effect of Ingestion of Alkali on Citric Acid in Blood and Urine

The experiment was performed with Dog 2, during a prolonged alkalosis period.

Blood		Urine		
Time	Citric acid	Citric acid	pH	Time
	mg. per 100 cc.	mg. per hr.		
10.00 a.m.	1.5	1.95	7.6	8.50 a.m.–10.50 a.m.
Basal diet + 20 gm. NaHCO₃ at 10.50 a.m.				
11.55 a.m.	2.0	13.8	8.0	10.50 a.m.–12.50 p.m.
1.50 p.m.	2.0	34.5	8.0	12.50 p.m.– 2.50 "
3.50 "	2.1	24.3	8.0	2.50 " – 4.50 "

sodium bicarbonate, although the equivalent amount of citrate as the free acid failed to cause excretion of "extra" citric acid. However, the largest amount of citrate excreted was equivalent to less than 1 per cent of that ingested, indicating that no extensive diminution in citric acid tolerance was associated with alkalosis of 2 days duration. The citric acid tolerance should be investigated in prolonged alkalosis, when, as pointed out above, the excretion of metabolic citric acid is enormously increased.

In order to determine whether the increased excretion of citrate in alkalosis is the result of a rise in the blood level, simultaneous determinations of citric acid in the blood and urine were made following the ingestion of alkali. Typical data for Dog 2 are given in Table X. Although it had been found that this individual

excreted "extra" citric acid in response to citric acid administration only when the apparent renal threshold of about 6.0 mg. per cent (see Fig. 2, Sherman *et al.* (1936)) was exceeded, ingestion of alkali caused a large increase in excretion of citrate with a relatively slight rise in blood level to 2.1 mg. per cent. This indicates that a rise in the blood level of citric acid cannot account for its increased excretion in alkalosis, and suggests that the kidney is specifically involved in the process. The diuresis produced by alkali administration is not the determining factor, since the production of the same volume of urine (2000 cc.) by forced fluid intake did not augment citrate excretion significantly. Östberg (1931) suggested that the urinary citrate, like the urinary ammonia, may originate in the kidney, but Kuyper and Mattill (1933), applying the Thunberg method to blood from the portal, hepatic, and renal veins and from the heart of a rabbit to which a single dose of bicarbonate had been given, noted a diminished, rather than an increased, citrate content in blood leaving the kidney. It is suggested that this experiment might well be repeated in animals in which a maximal excretion of citrate has been forced, and that in such experiments the colorimetric determination of citrate (Pucher *et al.*, 1936) be employed, as this is apparently more independent of the concentration of other constituents in the blood than is the Thunberg enzymatic method.

SUMMARY

Citric acid was invariably found in normal urine of human subjects, rats, and dogs, and in the blood, feces, and body tissues of the latter species.

The observations on man confirm the findings of Östberg that the amount of citrate excreted by a given individual varies directly with the urinary pH regardless of the cause of the alterations in the latter value.

The addition of sodium bicarbonate (10 per cent of the dry mixture) to the basal citrate-low diet of rats caused a 100-fold increase in citrate elimination.

In some dogs the excretion of citric acid was decidedly greater on a low protein, high sucrose diet than on a low carbohydrate, high casein diet, although in others dietary alterations produced no consistent change.

The ingestion of the basal, citrate-low diet generally produced an increase in the rate of citrate excretion, and in some, but not all, cases, a slight rise in the blood citrate concentration of dogs.

In dogs, repeated daily administration of alkali produced a 20- to 100-fold increase in citrate excretion, which was greatly favored by the substitution of sucrose for the casein of the basal diet, although the addition of sucrose *per se* produced no increase. The increased excretion cannot be attributed simply to a rise in the blood level of citrate, nor to the diuresis produced by alkali, and a specific activity of the kidney is suggested.

Consideration of the large amounts of citrate which are excreted by dogs on a citrate-low diet during prolonged alkalosis, and the absence of stores of preformed citrate in blood, liver, muscle, and kidney, lead to the conclusion that the dog can synthesize citric acid.

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THE METABOLISM OF ORALLY ADMINISTERED
CITRIC ACID*

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The increase in total organic acids of the urine following the ingestion of large amounts of citrus fruit juices has been generally interpreted as a measure of the amount of citric acid escaping oxidation in the body (Blatherwick and Long, 1922; Chaney and Blunt, 1925; Saywell and Lane, 1933). This is an unsatisfactory criterion for several reasons. By the Van Slyke and Palmer (1920) titration method for organic acids only a part of the citric acid present may be determined.[1] Furthermore, it has been demonstrated that the production of a more alkaline urine by the ingestion of fruit juices or of alkalies may, in itself, induce an augmented excretion of citric acid (Fasold, 1930; Östberg, 1931; Schuck, 1934) and of other organic acids (Booher and Killian, 1924; Haldane, 1924; Fasold, 1930). Finally, even when the addition of citric acid is the only dietary change, the "total organic acids" is not an accurate measure of the citric acid content of the urine.[2] Thus a

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* The data in this paper were taken from a dissertation presented by Caroline C. Sherman in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1935. A part of the expense of this investigation was borne by the William Gilman Thompson Fund and the Russell H. Chittenden Fund for Research in Physiological Chemistry.

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[1] The calcium hydroxide added as a preliminary step in the Van Slyke and Palmer procedure may cause considerable precipitation of citrate. The "total organic acids" titration value of a 0.1 per cent solution of citric acid was 40 per cent less after the calcium treatment than before, and direct determination of citric acid in the filtrate indicated a comparable loss.

[2] The following data for the pH, total organic acids, and citric acid content of the 24 hour urine collection of Dog 2 on control days and following

satisfactory estimate of the ability of the organism to destroy ingested citric acid requires not only a direct determination of the citrate reappearing unchanged, but also the exclusion of factors disturbing the acid-base equilibrium.

Gonce and Templeton (1930) observed no increase in the citric acid output of four normal children when 0.6 gm. of citric acid per kilo of body weight was given daily for 3 days. Of five normal individuals ingesting numerous daily doses of 10 to 40 gm. of citric acid, Östberg (1931) found that only one exhibited any considerable increase in urinary citric acid. The administration of the material to the other subjects caused slight variations in the citric acid excretion, in general paralleling changes in the urinary pH. Kuyper and Mattill (1933) reported that when 2 to 20 gm. of citric acid were administered either to fasting individuals or in addition to a constant diet, 1.5 to 2.5 per cent escaped oxidation. Three young women who ingested 12 gm. of citric acid daily for 8 days excreted no more citric acid than in a control period; whereas three others excreted "extra" citric acid corresponding to 1.6, 3.2, and 3.6 per cent of the acid ingested (Schuck, 1934). The hog has been reported to utilize completely more than 2.0 gm. of citric acid per kilo administered orally (Fürth *et al.*, 1934; Woods, 1927).

An increase in the serum concentration of citric acid of 0.7 to 1.5 mg. per cent was noted following the administration of 5 to 15 gm. of citric acid to normal human subjects, and to individuals with disturbed citrate metabolism (Thunberg, 1933; Lennér,

the administration of large doses of citric acid illustrate the lack of parallelism between the Van Slyke and Palmer titration figure and the citric acid content of the sample. The increase in the total organic acids following the ingestion of large amounts of citric acid is also to be noted.

Date	Citric acid ingested	pH	Total organic acids	Citric acid	
	gm. per day		*cc. 0.1 N acid*	*cc. 0.1 N acid*	*mg.*
Oct. 14	35.2	5.7	536	31	198
" 16	0	5.7	232	1.0	6
" 18	17.6	5.7	420	1.1	7
" 20	13.2	5.6	320	6.2	40
" 21	0	5.7	256	0.6	4
" 22	26.4	5.9	414	41	262
" 24	0	5.8	272	1.3	8

1934); similar blood studies on rabbits have been complicated by anesthesia, but have shown large rises in the blood level (to as high as 20 to 40 mg. per cent) (Salant and Wise, 1916–17; Kuyper and Mattill, 1933). There have been no reports of tolerance studies in which simultaneous determinations of the citric acid concentration in blood and urine of the same individual were made, which might give some insight into the question of the renal threshold for citrate.

The present study concerns the effect of the ingestion of large doses of citric acid by dogs on the amounts of the acid present in the urine, blood, and feces, and on the urinary pH, organic acid, and nitrogen excretion. In some instances, blood and urine determinations were made at frequent intervals in an attempt to establish the relationship between the concentration of citrate in the blood and its rate of excretion by the kidney.

EXPERIMENTAL

The animals used in this study were adult dogs of both sexes, weighing 8 to 22 kilos, which received a constant daily allowance of one of the basal diets described in the preceding paper (Sherman *et al.*, 1936). Solutions of citric acid were administered by stomach tube. With the exception of Dog 4, urine collections were made by catheterization. Blood was drawn from the jugular vein, oxalated, and the blood filtrates immediately prepared for analysis. Carmine suspended in the citric acid solution was used to mark the feces corresponding to the experimental period. Citric acid was determined by the method previously described (Pucher *et al.*, 1936), and the pH by an electrometric measurement with the antimony-antimony trioxide electrode (Roberts and Fenwick, 1928).

Following the ingestion of 8 to 35 gm. of citric acid (0.5 to 2.0 gm. per kilo of body weight), there occurred in some cases no increased excretion of citrate by the kidney, and in other cases elimination of as much as 450 mg. of citric acid in excess of the basal value (Table I). In twenty experiments of this sort, the excess citric acid in the urine corresponded, at most, to 5 per cent, and, on the average, to 0.7 per cent of the ingested material. The variations in the citric acid tolerance of the different animals were quite marked: thus, of four animals ingesting 0.5 gm. of citric

acid per kilo of body weight, one excreted none, a second 0.5 per
cent, a third 1.7 per cent, and the fourth, 5.0 per cent of the mate-
rial as "extra" citric acid in the urine.   At least 95 per cent of this
citrate which escaped oxidation was excreted within about 7 hours,
and the remainder usually within less than 24 hours.

The administration of citric acid to fasting dogs was followed
promptly (within less than half an hour) by a decided increase in

TABLE I

Effect of Ingested Citric Acid on Urinary Citric Acid

Dog No	Citric acid ingested		Citric acid in urine, excess over basal value	
	gm per kg. body weight	*gm.*	*mg.*	*per cent of a administered*
1	0.5	9.0	450	5.0
2	0.5	8.8	6.0	0.07
	1.0	17.6	5.5	0.03
	2.0	35.2	192	0.5
	1.0	17.6	0	0
	1.0	17.6	0	0
	1.5	26.4	0	0
	0.75	13.2	40	0.3
	1.5	26.4	256	1.0
	0.5	8.8	0	0
	1.0	17.6	170	1.0
3	1.0	8.0	0	0
4	1.0	16.0	40	0.3
	1.0	16.0	110	0.7
	1.0	16.0	30	0.2
	1.0	16.0	135	0.8
	1.0	16.0	90	0.6
	0.5	8.0	40	0.5
	1.0	16.0	80	0.5
5	0.5	11.0	190	1.7

the citrate concentration of the blood, which reached a maximum
of 2 to 4 times the basal level within $\frac{1}{2}$ to $3\frac{1}{2}$ hours and returned
practically to normal within $3\frac{1}{2}$ to $7\frac{1}{2}$ hours (Fig. 1).   The failure
of the citrate concentration of the general circulation to reach
more than 9 mg. per cent when as much as 17.6 gm. was ingested
and the comparatively short time required for the blood level and
urinary excretion to return to normal suggest a highly efficient
mechanism for the disposal of orally administered citric acid in the
dog.

Simultaneous determinations of citrate in blood and urine in hourly periods following oral administration of citric acid indicated the existence of a renal threshold for citrate in the dog. The
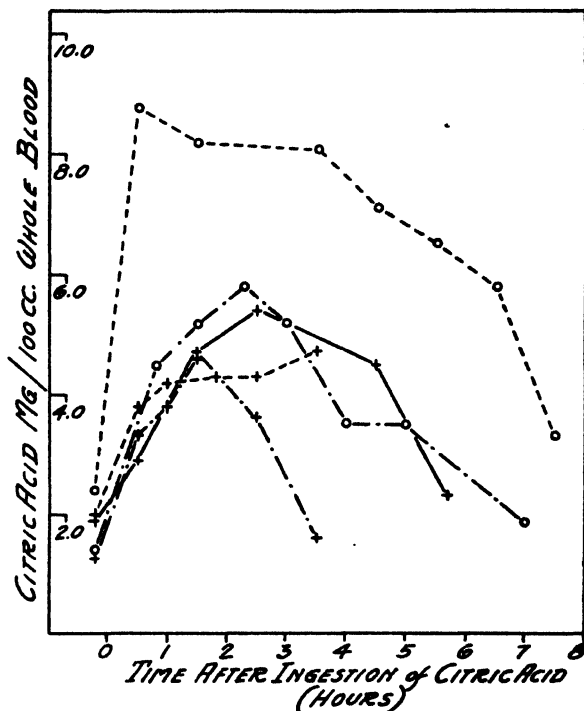
FIG. 1. The effect of ingestion of citric acid upon the concentration of citric acid in the blood. The data for Dog 1 are shown by the solid line; for Dog 2 by the dash line; for Dog 4 by the dash and dot line. The cross denotes 0.5 gm. of citric acid; the circle, 1.0 gm. of citric acid per kilo of body weight.

differences between two dogs in this respect are illustrated by Fig. 2. In the case of Dog 1, the urinary excretion of citrate was greatly increased when the concentration of citrate in the blood reached about 2.3 mg. per cent; with Dog 2, on the other hand, no increased excretion occurred when the blood level rose as high as 4.7 mg. per cent, and the kidney threshold was only exceeded when, with larger doses of the acid, blood citrate concentrations as great as 5.8 to 6.5 mg. per cent were attained. Such differences in the

renal threshold for citrate offer a probable explanation for the considerable individual variations in citric acid tolerance shown in Table I.

No increased amounts of citric acid were found in the feces when large doses of the substance had been administered. It is, of course, possible that a part of the ingested citric acid failed to be
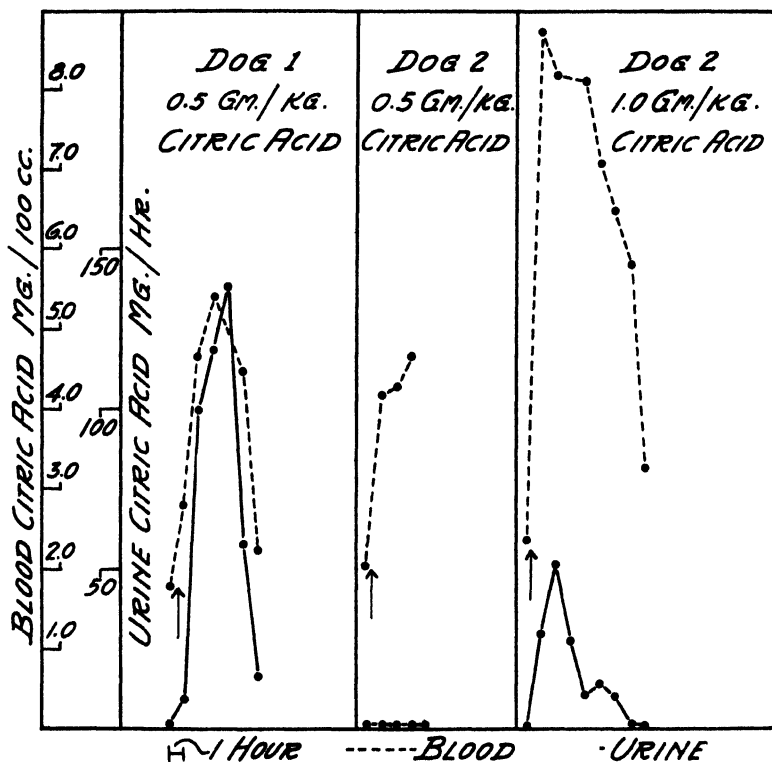
FIG. 2. The relation of the blood level to the urinary excretion of citric acid following the ingestion of citric acid.

absorbed and was destroyed by microorganisms in the gastro-intestinal tract.

The ingestion of large quantities of citric acid was without effect on the pH of the 24 hour collection of urine, although a temporary drop was occasionally noted in the pH of short time collections containing much "extra" citric acid.

No decrease in the urinary nitrogen, such as that reported by Fürth *et al.* (1934) for the hog, was observed when 0.5 or 1.0 gm. of citric acid per kilo of body weight was administered to dogs in nitrogen equilibrium or in negative nitrogen balance.

SUMMARY

The dog has the ability to destroy nearly completely large amounts of ingested citric acid. Following the oral administration of 0.5 to 2.0 gm. of citric acid per kilo of body weight, an average of 0.7 per cent of the acid given escaped oxidation and appeared in the urine; a rise in the blood citrate level was maintained for $3\frac{1}{2}$ to $7\frac{1}{2}$ hours; and no extra citric acid appeared in the feces.

. Apparent renal threshold values varying from about 2.2 to about 6.0 mg. of citric acid per 100 cc. of whole blood have been observed.

Ingestion of citric acid in addition to a constant diet did not affect the pH or the total nitrogen of the 24 hour urine collection.

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NITROGEN SOLUBILITY IN BLOOD AT INCREASED AIR PRESSURES

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In a series of studies to determine the effect of increased air pressure on the human body it seemed necessary to determine ʼwhether nitrogen is taken up, at greatly increased pressures, according to the laws governing the solution of gases. This is important, as men who have been working in compressed air, either in diving suits or caissons, are apt to develop compressed air illness. This illness is considered to be due to the fact that gas (chiefly nitrogen) which goes into solution in the blood and tissues during such exposure is subsequently liberated in the form of bubbles following too rapid decompression. These bubbles produce local or general blockage of the circulation or other injury.

Conant and Scott (1926) studied nitrogen solubility in blood at various nitrogen tensions from atmospheric downwards and found the amounts of nitrogen dissolved did not follow Henry's law, and interpreted their results as evidence that nitrogen was absorbed by the hemoglobin. Van Slyke, Dillon, and Margaria (1934), using special precautions against technical error, showed that nitrogen solubilities in this zone of tensions are in agreement with Henry's law. Since neither of these studies was made at nitrogen tensions above atmospheric, this series of experiments with nitrogen tensions varying up to and including 6 atmospheres (absolute) was conducted.

EXPERIMENTAL

Ox blood was collected from the jugular vein of calves.

Dog blood was collected from the cannulated femoral artery of dogs anesthetized with nembutal.

The blood was oxalated and either used immediately or chilled to nearly 0°, and kept in tightly stoppered flasks at that temperature in the ice box until samples were saturated the following morning. Sufficient oxalate was added to make a 0.02 per cent solution.

The solubility of nitrogen in water at 1, 2, 4, and 6 atmospheres was determined to control the accuracy of the methods used.

Equilibrations under increased pressures were conducted in a recompression chamber described by Hawkins, Shilling, and Hansen (1935) by the use of a portable water bath.

A mercury manometer was connected to the recompression chamber so that pressures could be read accurately.

Method of Saturation

Saturation of the liquids with nitrogen gas from a metal cylinder was carried out in a double tonometer according to the first saturation method of Austin *et al.* (1922). A 15 cc. tonometer was connected with a 1000 cc. tonometer by means of a short, large bore, rubber tube to make a double tonometer. The smaller vessel was filled with the liquid to be saturated, while the larger vessel was completely exhausted, washed out, and filled with nitrogen gas. The liquid was then run into the larger vessel and the tonometer was rotated horizontally in a constant temperature water bath maintained at 38° ± 0.05°. The desired pressure was obtained by opening, at intervals, under water, the capillary stopcock of the larger vessel, thus permitting the pressure of the gas, raised by warming in the bath, to come to the various pressures at which the equilibrations were being made. The gas was not renewed because of the limited time that the operators could stay at the increased pressures. The total time allowed for saturation of the liquid was 30 minutes. Equilibration was apparently attained in this time.

After saturation the separation of the liquid phase in the small tonometer and the gas phase in the large tonometer was carried out as described by Austin *et al.* (1922). Both the gas and the liquid phases were analyzed within 1 hour by the following methods.

The N₂ tensions were determined as described in the saturation method of Austin *et al.* (1922), by analysis, by Haldane's method,

of the separated gases in the upper chamber of the tonometer. At increased pressures, this chamber was attached to a leveling bulb filled with mercury and some of the gas forced out to allow for the expansion of the remaining gas when the pressure was reduced to atmospheric.

The nitrogen tension in the gas phase was calculated from the nitrogen content by the usual equation

$$P = \frac{\text{per cent N}_2 \text{ in gas phase}}{100} \times (\text{manometer} - W)$$

where manometer is the corrected manometer pressure and W is the vapor tension of water at $38°$ (W 49.7 mm.).

The total N_2 contents of the solutions were determined by means of the manometric apparatus of Van Slyke and Neill (1924), the N_2 content being calculated as

$$\text{Volume per cent nitrogen} = P \times N_2 \text{ factor}$$

At atmospheric pressures 5 cc. samples were taken for analysis when the water or blood was equilibrated. Pressure readings for the measurement of the extracted nitrogen gas were made after bringing the gas to a 0.5 cc. volume. CO_2 and O_2 were absorbed by air-free Fieser's hydrosulfite solution (1924), and the remaining gas measured as nitrogen. Duplicate analyses were made on both the liquid and gas phases of the equilibrated blood or water.

At the increased pressures where equilibration was made the blood or water to be analyzed was transferred from the tonometer to a special pipette with a 3-way stop-cock similar to that described by Van Slyke and Neill (1924). It was necessary to use this type of pipette to prevent any loss of N_2 as it escaped from the supersaturated solutions while the pressures were being reduced to normal. The pipette had a small bulb between the calibrated bulb and the lower stop-cock to prevent the liquid, on the expansion of gas, being driven down into the rubber tubing connecting the pipette with the mercury leveling bulb.

After return to atmospheric pressure the total gas and liquid contents of the pipette were delivered quantitatively to the manometric apparatus.

Oxygen capacity determinations were made on blood to determine hemoglobin content (Van Slyke and Neill (1924)).

The water content of the bloods was determined by drying 2 cc. samples to constant weight at 110°.

The solubility coefficients α and α_0 were calculated as described by Van Slyke, Sendroy, Hastings, and Neill (1928).

The results are given in Tables I and II.

Water saturated at 38°, with nitrogen pressures varying from 1 to 6 atmospheres (absolute), was found by analysis to contain the amounts of nitrogen which would be calculated from Henry's law.

$$\text{Volume per cent dissolved } N_2 = \frac{100 \, \alpha \, p}{760}$$

TABLE I

Nitrogen Solubility at 38° in Water

Experiment No.	N$_2$ tension at 38°	N$_2$ content of water	α
	mm.	*vol. per cent*	*cc. N$_2$ per cc. solution*
1	714	1.20	0.01270
	714	1.22	0.01292
2	718	1.19	0.01255
	718	1.20	0.01263
3	1486	2.52	0.01281
	1486	2.59	0.01317
4	2980	4.95	0.01256
	2980	4.99	0.01266
5	4528	7.48	0.01249
	4528	7.44	0.01243
6	4592	7.68	0.01271
	4592	7.67	0.01269

The results for the solubility coefficient of nitrogen in water, determined by saturation at 1 to 6 atmospheres (absolute) agree closely with those of earlier authors.

The limits of error of the technique used are shown in Table I by the duplicate nitrogen volume per cent determinations and duplicate saturations. The greatest deviation between the duplicate samples was 0.07 volume per cent and this in only one case. The maximum deviation from the average solubility of nitrogen in water was 0.04 volume per cent.

The constancy in analyses of water shows that the technique, when used on blood, is sufficiently accurate to determine whether the amounts of nitrogen dissolved in blood at increased pressures follow Henry's law.

Table II shows the nitrogen solubility coefficient and the amounts of N_2 in volume per cent dissolved in ox and dog bloods equilibrated with N_2 pressures from 1 to 6 atmospheres (absolute).

TABLE II

Nitrogen Solubility at 38° in Blood

Sample No.	O_2 capacity	H_2O content	N_2 tension at 38°	N_2 content of blood	α	α_0
	vol. per cent	gm. per cc.	mm.	vol. per cent	cc. N_2 per cc. solution	cc. N_2 per gm. H_2O
D-1	20.14	0.8069	715	1.31	0.0138	0.0171
			1481	2.68	0.0136	0.0169
			3071	5.52	0.0136	0.0169
			4508	8.04	0.0134	0.0166
D-2	20.06	0.8965	701	1.31	0.0141	0.0157
			1460	2.75	0.0142	0.0158
			3006	5.39	0.0135	0.0151
			4524	8.26	0.0138	0.0154
D-3	21.55	0.7767	712	1.31	0.0139	0.0179
			1471	2.48	0.0127	0.0164
			3006	5.45	0.0137	0.0176
			4434	7.92	0.0135	0.0174
D-4			698	1.37	0.0148	
			1474	2.85	0.0146	
			4494	8.46	0.0142	
O-1	15.70	0.8223	567	1.01	0.0135	0.0164
			1562	2.73	0.0133	0.0162
			2913	4.82	0.0126	0.0154
O-2	18.18	0.8113	703	1.31	0.0140	0.0173
			1510	2.75	0.0137	0.0169
			2936	5.12	0.0132	0.0163
			4215	7.35	0.0133	0.0164
O-3	18.50	0.8079	699	1.24	0.0134	0.0166
			2960	5.20	0.0132	0.0163
			4472	7.55	0.0128	0.0158
O-4	20.01	0.8799	701	1.23	0.0132	0.0150
			2967	5.19	0.0132	0.0150
			4544	7.44	0.0124	0.0141
O-5	18.86	0.8122	698	1.23	0.0133	0.0164
			4461	7.80	0.0133	0.0164

The nitrogen solubility coefficient of the same blood equilibrated at pressures from 1 to 6 atmospheres (absolute) was found to be the same within the limits of experimental error.

The amount of N_2 dissolved in blood under N_2 pressures at 1, 2,

4, and 6 atmospheres (absolute) has been found directly proportional to the nitrogen pressure. These results agree with those of Van Slyke, Dillon, and Margaria (1934) who found that nitrogen was dissolved in blood and hemoglobin solutions under nitrogen pressures, varying from atmospheric downward, in accordance with Henry's law.

SUMMARY

The solubility coefficient of nitrogen in whole blood of normal dogs, equilibrated at atmospheric pressure, was found to vary from 0.0138 to 0.0148, and in ox bloods from 0.0135 to 0.0140.

The amount of nitrogen dissolved by whole blood under nitrogen pressures varying from 1 to 6 atmospheres (absolute) has been found directly proportional to the nitrogen pressure, according to Henry's law.

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THE DETERMINATION OF p-BROMOPHENYLMERCAP-TURIC ACID IN THE URINE OF THE DOG

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The extent of the synthesis of mercapturic acids in animals is usually estimated by studying the partition of urinary sulfur or by isolating mercapturic acids from the urine of animals which were fed halogenated benzenes. The former procedure is based on the assumption that the rise in the output of neutral sulfur in the urine of animals on the day following the administration of benzene derivatives is due entirely to the excreted mercapturic acids. This assumption lacks experimental justification. The isolation of mercapturic acid, when used as a sole criterion of its presence in the urine, has led to contradictory interpretations of the results obtained, since an isolation procedure such as that of Baumann and Schmitz (1), most commonly used, is not only far from being quantitative, but also fails to detect small amounts of mercapturic acid in the urine (*cf.* 2–5). The need for a quantitative method for estimating mercapturic acids in the urine has been emphasized on several occasions (6). An attempt to devise such a method was made by McGuinn (7), but no quantitative procedure was worked out.

We desired a comparatively simple, reasonably accurate method, requiring small volumes of urine, in order that the determination of urinary sulfur partitions, mercapturic acid, and isolation of the mercapturic acid could be made on the same sample of urine. The method presented here is believed to fulfil these requirements and offer a means of comparison between the rise in the output of neutral sulfur and the amount of mercapturic acid present in the urine after the administration of bromobenzene.

Reagents

1. Iodine in potassium iodide (0.01 N). 1.2692 gm. of resub-

limed iodine and 2 gm. of potassium iodide in 1000 cc. of distilled water.   The solution is standardized against 0.02 N sodium thiosulfate.

2. Sodium thiosulfate (0.02 N).   Standardized by the use of either potassium dichromate or potassium iodate.

3. Sodium hydroxide (2.5 N).

4. Sulfuric acid (2.5 N).

5. Zinc sulfate (10 per cent).

6. Sodium hydroxide (0.5 N).

7. Starch indicator (2 per cent solution of soluble starch in a saturated solution of sodium chloride).

8. Mercuric chloride (5 per cent).

9. Ethyl alcohol (95 per cent).

10. Ethyl ether.

Removal of Interfering Substances from Urine—The 24 hour sample of dog or pup urine is filtered and made up to 500 cc. with distilled water.   To 50 cc. of the diluted urine, 10 cc. of 10 per cent $ZnSO_4$ are added with continuous shaking, followed by 10 cc. of 0.5 N NaOH, added dropwise during shaking.   The mixture is allowed to stand for a few minutes and then filtered through a dry filter.[1]

Determination of the Mercapturic Acid by Use of $HgCl_2$—25 cc. of the clear filtrate are transferred to a 250 cc. Erlenmeyer flask. 4 cc. of 2.5 N NaOH are added, the flask is covered with a small watch-glass, and placed on a hot-plate at "low" heat for 25 to 30 minutes.   After this time, the flask and its contents are thoroughly cooled, first under the tap and then in an ice bath, and 10 cc. of 95 per cent alcohol are added and the liquids mixed.   To the mixture, 5.6 cc. of 2.5 N H_2SO_4 are added, followed, after the flask has been

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[1] Highly pigmented urines, collected from dogs fed mixed and high protein diets, show rather high absorption of iodine.   In this case, it is advisable to increase the amounts of $ZnSO_4$-NaOH used for the removal of interfering substances.   The proportions given here were used by us on our dogs.   It is probable that individual variations in dogs would demand an increase in the amounts of $ZnSO_4$ and NaOH to remove most of the interfering substances.   It is essential that the amounts of $ZnSO_4$ and NaOH used be such that the filtrates of normal urines show the same iodine blank before and after the alkaline hydrolysis of the filtrates.   In any case, the amount of 10 per cent $ZnSO_4$ added to the urine must be exactly the same as that of 0.5 N NaOH.   Of course, proper corrections for the increased dilutions must also be applied in the final calculation.

whirled, by 1 to 2 cc. of 5 per cent $HgCl_2$. The precipitate, which forms at once, is immediately filtered through a weighed Gooch crucible, washed with water, 95 per cent alcohol, and finally with ether, dried *in vacuo*, and weighed.

Calculation—1 gm. of the mercury complex of *p*-bromophenylmercaptan is equivalent to 1.1 gm. of *p*-bromophenylmercapturic acid. If 25 cc. of the $ZnSO_4$ = NaOH filtrate of the urine, prepared as described above, are used, the calculation is as follows: $a \times (500/17.86) \times 1.1$ = *p*-bromophenylmercapturic acid in gm. per 24 hour sample of urine, where a is the weight of $Hg(SC_6H_4Br)_2$ obtained from 25 cc. of $ZnSO_4$-NaOH filtrate.

Determination of Mercapturic Acid by Use of Iodine

Blank Titration—10 cc. of the $ZnSO_4$-NaOH filtrate are placed in a 250 cc. Erlenmeyer flask and cooled in an ice-salt bath; 10 cc. of 95 per cent alcohol are added, followed by 15 cc. of distilled water and 1.6 cc. of 2.5 N H_2SO_4 and 10 drops of starch solution. The mixture is cooled in an ice-salt bath, then titrated directly with a standard iodine solution added from a microburette until the blue color persists for at least 30 seconds. If desired, a definite volume of iodine solution may be added and the excess iodine titrated with standard thiosulfate solution. Either procedure was found satisfactory, yielding identical values. If direct titration with iodine is preferred, the standard solution must be checked from time to time by titration with standard thiosulfate and proper corrections applied, if necessary. As 0.02 N thiosulfate solution does not keep well, this solution was made up daily before use from 0.1 N thiosulfate by proper dilution.

Final Titration—10 cc. of the $ZnSO_4$-NaOH filtrate are placed in a 250 cc. Erlenmeyer flask, 4 cc. of 2.5 N NaOH and 15 cc. of distilled water are added, and the flask is covered with a watchglass and placed on a hot-plate, at "low" heat, for 25 to 30 minutes. The flask is then thoroughly cooled under the tap and then in an ice-salt bath. 10 cc. of 95 per cent alcohol are now added, followed by 5.6 cc. of 2.5 N H_2SO_4 and 10 drops of starch solution. The contents are kept in an ice-salt bath throughout the manipulation. The mixture is then titrated by either of the procedures described in the blank determination.

Calculation—1 cc. of 0.01 N iodine solution is equivalent to 3.18

mg. of *p*-bromophenylmercapturic acid.   If 10 cc. of the ZnSO₄-
NaOH filtrate, prepared as described above, are used, the calcula-
tion is as follows: $(a - b) \times (500/7.14) \times 3.18 = p$-bromophenyl-
mercapturic acid in mg. per 24 hour sample of urine, where a
represents cc. of 0.01 N iodine used in the final titration and b
is the cc. of 0.01 N iodine used in the blank titration.

<div align="center">DISCUSSION</div>

The determination of *p*-bromophenylmercapturic acid by the
use of $HgCl_2$ is based on the following reactions:

$$BrC_6H_4S\!-\!\overset{\overset{\displaystyle H_2}{\|}}{C}\!-\!\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle NH\!-\!CO\!-\!CH_3}{|}}{C}}\!-\!COOH \xrightarrow[\;H_2O\;]{NaOH} BrC_6H_4SH + NH_3 + CH_3COOH \atop \qquad\qquad\qquad + CH_3COCOOH \qquad (1)$$

$$2BrC_6H_4SH + HgCl_2 \longrightarrow \overset{\overset{\displaystyle S\!-\!C_6H_4Br}{|}}{\underset{\underset{\displaystyle S\!-\!C_6H_4Br}{|}}{Hg}} + 2HCl \qquad (2)$$
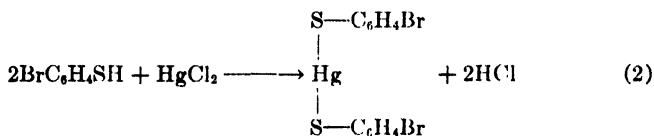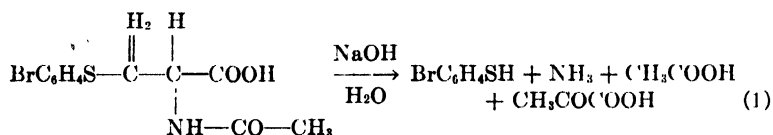
Reaction 1 was first described by Baumann (8).   Reaction 2 was
investigated in the following experiment performed on several
occasions, yielding almost identical results.   0.10 gm. of *p*-bromo-
phenylmercapturic acid, prepared from dog urine, was dissolved
in 100 cc. of distilled water and 16 cc. of 2.5 N NaOH, and heated
on a hot-plate at "low" heat for 25 to 30 minutes.   The solution
was then cooled in an ice-salt bath, and 40 cc. of 95 per cent alcohol
were added, followed by 22.4 cc. of 2.5 N H_2SO_4.   The flask was
whirled, and 3 cc. of 5 per cent $HgCl_2$ were added.   The precipi-
tate was centrifuged off, washed successively with water, 95 per
cent alcohol, and ether, and finally dried *in vacuo*.   The yield was
0.110 gm., or 100 per cent of the theoretical (calculated on the
basis of Reactions 1 and 2).   Analysis of the compound gave 35
per cent Hg and 11.3 per cent S.   Calculated for $Hg(S\!-\!C_6H_4Br)_2$,
34.7 per cent Hg and 11.15 per cent S.   The substance was
analyzed for Hg by dissolving the mercury compound in fuming
nitric acid under a reflux with gentle heat, evaporating the solution
nearly to dryness on a water bath, followed by the addition of

water, and precipitation of the Hg as iodate as described by Spacu and Spacu (9). The mercury complex obtained by the precipitation of p-bromophenylmercaptan (synthesized according to Hübner and Alsberg (10)) with $HgCl_2$ from acidified alcoholic solution gave nearly identical analytical results; *i.e.*, 34.3 per cent Hg and 11.15 per cent S. Thus it seems that the Reaction 2 is essentially correct.

Titration of the p-bromophenylmercaptan by iodine was suggested to us by the work of Klason and Carlson (11), who successfully determined p-thiocresol and p-thionaphthol by iodine titration of alcoholic solutions of these mercaptans, on the basis of Reaction 3.

$$2R—SH + I_2 \rightarrow R—S—S—R + 2HI \tag{3}$$

When precipitation with $HgCl_2$ and iodine titration were applied to dog urine, it was found that the urines gave small but consistent precipitates with $HgCl_2$ in acid medium and iodine absorption by normal urines was also rather high. The absorption of iodine by normal urines was previously observed by Fürth (12) and Lewis (13) and their coworkers. The use of charcoal for the removal of the interfering reducing substances, as suggested by Virtue and Lewis (13), was found to be unsuitable, since p-bromophenylmercapturic acid, similar to other mercapturic acids (14), is adsorbed by charcoal. Since $HgCl_2$ reacts with creatinine, uric acid, pyruvic acid, SH— compounds, etc., by combining with them or by being reduced, the use of $ZnSO_4$-NaOH, as used by Somogyi (15) for the removal of interfering reducing substances for glucose determinations in blood, seemed promising. The use of $ZnSO_4$-NaOH proved to be satisfactory for our purpose. $HgCl_2$ produced no precipitate with the acidified $ZnSO_4$-NaOH filtrate of normal dog urine within the time allowed to carry out the filtration and washing of the mercury precipitate, as described in the foregoing procedure. Faint turbidity, however, appears on prolonged standing, even in $ZnSO_4$-NaOH filtrates, when $HgCl_2$ is added as directed in the procedure. The interference with the determination by this extraneous matter which appears on prolonged standing is, however, improbable, since the determination is usually completed before any turbidity due to interfering matter can possibly develop. It seems unlikely that the turbidity which

develops on prolonged standing can be due to a slower secondary reaction between *p*-bromophenylmercaptan and $HgCl_2$.

Absorption of iodine by the acidified $ZnSO_4$-NaOH filtrate is reduced to one-third or less of the value obtained with normal acidified urines. We were unable to eliminate completely all the reducing substances which titrate with iodine. This necessitated making a blank determination on each $ZnSO_4$-NaOH filtrate before subjecting it to alkaline hydrolysis, and subtracting the value found from the titer obtained after the alkaline hydrolysis. Blanks on $ZnSO_4$-NaOH filtrates of normal urines containing no mercapturic acid were found to be the same before or after alkaline hydrolysis.[1]

As has been pointed out by Lucas and King (16) and Virtue and Lewis (13), in the iodine titration of cysteine, it is important to keep the temperature of the reacting mixture low and to use a rather high concentration of acid. They suggest an approximately 2 per cent acid concentration. In our method, the concentration of the acid is about 1.0 per cent. Lucas and King (16) have pointed out that in the case of aromatic mercaptans, such as thiophenol or thiocresol, the iodine absorption was constant between pH 0 and 7. We have also found that exact control of the acidity is not essential for the accuracy of the method, using either procedure. Although Lucas and King (16) have found that aromatic mercaptans can be safely titrated at room temperature, we preferred to carry out all determinations at 0°.

The procedure with $HgCl_2$ yields slightly higher results than those given by the iodine titration method. The consistency of the two procedures, however, in spite of these differences, serves as a satisfactory check on the procedure for the determination of mercapturic acid as a whole. As will be discussed in greater detail in our next report, under certain dietary conditions the rise in the output of neutral sulfur of the urine after feeding bromobenzene corresponds almost exactly to the output of mercapturic acid as determined by either of the procedures outlined above. Such a relationship, however, was not always found to hold true, especially in those cases where *l*-cystine, cysteine, *dl*-methionine, and taurine were administered with the bromobenzene over a period of several days. It was found that the rise in the output of neutral sulfur of the urine, under these conditions, was invariably con-

TABLE I

Recovery of p-Bromophenylmercapturic Acid from Water and Urine

Medium	Mercapturic acid added	0.01 N I₂ absorbed			Hg(SC₆H₄Br)₂	Mercapturic acid found by methods		Recovery
		Blank (b)	Final (a)	(a − b)		I₂	HgCl₂	
	mg.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	*per cent*
Water	10.0				8.7		9.6	96
"	10.0				9.0		9.9	99
"	6.0				5.5		6.1	101
"	10.3				10.1		11.1	107
"	12.0	0	3.64	3.64		11.60		97
"	10.3	0	3.13	3.13		9.96		97
"	10.3	0	3.26	3.26		10.37		101
"	12.0	0	3.71	3.71		11.80		98
Pup urine	10.0				8.9		9.8	98
" "	10.0				9.6		10.6	106
" "	10.0	0.70	3.88	3.18		10.10		101
" "	10.0	0.70	3.84	3.14		10.00		100
Dog "	10.0				9.4		10.3	103
" "	10.0				9.3		10.2	102
" "	10.0				9.1		10.0	100
" "	10.0				9.6		10.6	106
" "	4.1	0.60	1.83	1.23		3.91		95
" "	4.1	0.60	1.93	1.33		4.20		102
" "	4.1	0.60	1.84	1.24		3.97		97
" "	4.1	0.60	1.89	1.29		4.11		100
" "	10.0*				9.4		10.3	103
" "	10.0†				9.5		10.4	104
" "	10.0‡				8.8		9.7	97
" "	10.0§				9.3		10.2	102
" "	10.0*	0.63	3.71	3.08		9.80		98
" "	10.0†	0.60	3.71	3.11		9.90		99
" "	10.0‡	0.60	3.77	3.17		10.10		101
" "	10.0§	0.61	3.69	3.08		9.80		98

The recoveries were made on 25 cc. of water and ZnSO₄-NaOH filtrate of urine with the HgCl₂ procedure, and on 10 cc. of water and ZnSO₄-NaOH filtrate of urine with the I₂ procedure. The mercapturic acid was dissolved in urine before the addition of ZnSO₄-NaOH.

* Urine collected after repeatedly feeding *l*-cystine (1.0 gm. per day).

† Urine collected after repeatedly feeding cysteine hydrochloride (1.54 gm. per day).

‡ Urine collected after repeatedly feeding *dl*-methionine (1.26 gm. per day).

§ Urine collected after repeatedly feeding taurine (1.08 gm. per day).

TABLE I—*Concluded*

Medium	Mercapturic acid added	0.01 N I₂ absorbed			Hg(SC₆H₄Br)₂	Mercapturic acid found by methods		Recovery
		Blank (b)	Final (a)	(a − b)		I₂	HgCl₂	
	mg.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	*per cent*
Pup urine...	10.0*				9.2		10.1	101
" " ...	10.0†				9.1		10.0	100
" " ...	10.0‡				9.4		10.3	103
" " ...	10.0§				9.6		10.6	106
" " ...	10.0*	0.73	3.81	3.08		9.80		98
" " ...	10.0†	0.71	3.85	3.14		10.00		100
" " ...	10.0‡	0.70	3.87	3.17		10.10		101
" " ...	10.0§	0.70	3.75	3.05		9.70		97
" " ...	‖	39.00	224.2	185.2	540	589	594	
" " ...	‖	40.00	216.1	176.1	510	560	561	
Dog " ...	‖	36.00	259.9	223.9	724	712	797	
" " ...	‖	36.60	269.3	232.7	706	740	777	

‖ Urine collected after a single feeding of 1.0 gm. of bromobenzene. These results are expressed per 24 hour sample of urine.

siderably higher than the output of mercapturic acid sulfur. *l*-Cystine, cysteine, taurine, and *dl*-methionine, when *fed alone repeatedly* to the same dog, increased the output of neutral sulfur of the urine. The recovery of *p*-bromophenylmercapturic acid added to such urines, as is shown in Table I, was, however, satisfactory, thus indicating that the presence of unchanged taurine, or of partially oxidized *l*-cystine, cysteine, or methionine does not interfere with the determination of *p*-bromophenylmercapturic acid in the urine.

Table I summarizes the experiments in which the recovery of *p*-bromophenylmercapturic acid from water and the urines of several dogs maintained on diets of various sulfur contents was determined by both of the procedures presented here. The recoveries seem satisfactory enough to warrant the application of the method for metabolic studies on dogs. Several determinations of *p*-bromophenylmercapturic acid in the urine of dogs which were fed bromobenzene are illustrated in Table I.

Pending the application of the procedure to urines of other animals, we suggest that the method as presented here be reserved

for the urines of dogs and pups only. Inasmuch as other halogen-mercapturic acids and *l*-α-naphthalenemercapturic acid are essentially similar to *p*-bromophenylmercapturic acid, in so far as they all yield aromatic mercaptans on alkaline hydrolysis, it seems probable that the method as outlined here is also applicable for the determination of these mercapturic acids in dog urine. However, further work is necessary in order to check such a possibility.

We realize that the reactions on which our method is based are not specific in nature and therefore limit the value of the method. However, under the conditions under which the extent of the synthesis of mercapturic acid in dogs is generally studied, the method outlined seems more reliable in its nature than quantitative interpretation of the fluctuation of the neutral sulfur in the urine after feeding bromobenzene under varying dietary conditions.

SUMMARY

A method for the determination of *p*-bromophenylmercapturic acid in dog urine by two procedures is presented.

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COMPOSITION OF PATHOLOGICAL CALCIUM DEPOSITS*

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The chemical composition of certain pathological calcium deposits has been studied but little. There are a few analyses of the calcific material from sclerotic aortas (Barillé (1), Schoenheimer (2)), but Wells (3) was unable to find a report of any chemical study of Mönckeberg's sclerosis of the larger muscular arteries. Furthermore, no analyses have been published of the composition of the calcium deposits which form following the administration of large doses of viosterol to rabbits. This report is intended to fill these gaps, at least partially. It comprises the results of analyses for calcium, phosphorus, and, where possible, magnesium, of (a) isolated calcified plaques from the media of muscular arteries, (b) isolated calcified plaques from the aorta, (c) small masses of calcific material laid down in various sites in rabbits given toxic doses of viosterol. These deposits were formed in the animal's own aorta and in pieces of sterile rabbit aorta and of agar jelly which had been implanted subcutaneously and intraperitoneally.

Mönckeberg's Sclerosis—Calcified plaques were dissected from the media of several iliac arteries, the bifurcation of an aorta, and a uterine artery. The microscopic examination of part of each plaque confirmed the medial location of the calcific deposit. The tissues had been preserved for some time in Klotz' and Kaiserling's solutions. The plaques were cleaned, ground in a mortar, and dried to constant weight in an oven at 110°. The material was then ashed, essentially as described by Gabriel and by Schoenheimer (2), with 3 per cent KOH in glycerol at 170° for 5 hours. The ash was washed once with water, dried, and the ashing re-

peated.   After the second ashing, the residue was washed with
distilled water until the washings were no longer alkaline.   (The
ashing was carried out in a 15 cc. conical Pyrex centrifuge tube to

TABLE I

Analysis of Medial Calcified Plaques (Mönckeberg's Sclerosis)

Autopsy No.	Source of material	P_2O_5	CaO	MgO	Ca : P
		per cent	per cent	per cent	
11078	Iliac artery	36.9	49.7	1.04	2.20
11494	" "	36.2	48.2		2.18
11261	Bifurcation of aorta	36.0	47.8	0.88	2.17
10872	Uterine artery	36.9	49.1	0.98	2.19
10804	Iliac artery	36.9	48.6		2.16
10570	" "	38.0	49.8		2.14
Average....................		36.8	48.9	0.97	2.17

TABLE II

Analysis of Intimal Calcified Plaques (Aortic Sclerosis)

Autopsy No.	P_2O_5	CaO	MgO	Ca : P
	per cent	per cent	per cent	
11717	36.6	50.5	0.86	2.21
11735	35.7	51.3	0.86	2.35
11738	35.3	50.3	0.86	2.33
X*	35.9	50.5	0.86	2.29
11708	35.5	50.6	0.99	2.34
11655	35.7	50.4	0.96	2.31
11152	35.3	51.0	0.93	2.36
10807†	36.2	47.5	0.85	2.14
11494†	35.8	50.0		2.19
10570†	36.2	48.1	0.67	2.17
11078†	36.7	49.4	1.01	2.20
10804†	34.4	48.6		2.31
Average....	35.8	49.8	0.87	2.27

* Combined small plaques from four aortas.
† Not defatted.

facilitate washing.)   The residue was then dried to constant weight
and dissolved in N HCl for analysis.   Calcium was determined by
the modified method of Halverson and Bergeim (4), phosphorus
by that of Fiske and Subbarow (5), and magnesium by that of

Briggs (6). Determination of the CO_2 content of the limited quantities of material was not practicable.

The results of the analyses are given in Table I.

Aortic Sclerosis—Calcified plaques from fixed human sclerotic aortas were subjected to the same manipulations described above. In addition to mechanical cleaning of the plaques some of them were defatted by repeated treatment with hot alcohol and ether.

Table II contains the results of the analyses of plaques from twelve sclerotic aortas.

Calcium Deposits Due to Viosterol—This portion of the work had a twofold purpose: (1) to determine whether an inert colloidal gel or dead tissue from another animal implanted subcutaneously or intraperitoneally would serve as a framework for the deposition of calcium salts under the stimulus of large doses of viosterol and the attendant hypercalcemia; (2) to ascertain the composition of such calcium deposits, if any occur.

A 3 per cent agar solution in distilled water was sterilized and permitted to set in cylindrical forms (test-tubes) approximately 1.3 cm. in diameter and 4 cm. long. 3 cm. lengths of fresh normal rabbit aortas were sterilized by boiling. A cylinder of agar and a length of aorta were inserted aseptically into the peritoneal cavity and also into subcutaneous abdominal pockets of each of four adult rabbits. Weekly subcutaneous injections of 10,000 D viosterol were begun immediately.[1] Two of the animals received 0.25 cc. per kilo and 2 received 0.5 cc. per kilo weekly for three injections, then 0.3 cc. per kilo and 0.75 cc. per kilo respectively for two injections. Only a slight rise in blood calcium followed this treatment. Accordingly, a different 10,000 D viosterol preparation was substituted, and all the rabbits received 1 cc. per kilo weekly for the remaining 6 weeks of the experiment, by which time they were toxic and emaciated. Meanwhile two additional similar operations had been performed on each rabbit at monthly intervals, so that when the animals were killed, approximately 3 months from the beginning of the experiment, each had received three pieces of agar and three pieces of aorta intraperitoneally and subcutaneously. None of these was removed until the end of the experimental period.

One rabbit died 7 weeks after the first operation. No gross

[1] We are indebted to E. R. Squibb and Sons and to Mead Johnson and Company for the 10,000 D viosterol preparations.

TABLE III

Analysis of Calcified Material from Rabbis Given Viosterol and from Controls Given No Viosterol

Rabbit No.	Source of material	Time in animal	Gross findings	P₂O₅	CaO	Ca : P
		mos.		*per cent*	*per cent*	
	Rabbits given viosterol					
197	Agar mass implanted intraperitoneally	1	Ca in capsule only	27.2	51.7	3.10
	" " "	2	" " agar and capsule	33.4	46.0	2.25
	" " "	3	" " " "	33.2	47.3	2.32
	Aorta implanted intraperitoneally	2	Few Ca granules	28.2	42.2	2.45
	" "	3	Diffusely calcified	34.2	51.0	2.43
	Agar masses implanted subcutaneously		No calcification			
	Aorta implanted subcutaneously	2	Few Ca granules	32.0	44.5	2.27
	" "	3	" " "	33.4	47.1	2.30
	Rabbit's own aorta		Extensively calcified	30.9	40.3	2.13
199	Agar mass implanted intraperitoneally	2	Ca in capsule only	31.0	45.2	2.32
	" "	3	" " "	32.7	45.8	2.32
	Aorta implanted intraperitoneally	2	Few Ca granules	28.9	44.3	2.51
	" "	3	" " "	33.2	49.0	2.41
	Agar masses implanted subcutaneously		No calcification			
	Aorta implanted subcutaneously	2	Few Ca granules	32.3	46.4	2.35
	" "	3	" " "	30.7	44.3	2.36
	Rabbit's own aorta		Extensively calcified	33.0	46.2	2.26
	Rib			37.6	50.0	2.17

201	Agar mass implanted intraperitoneally	2	Ca in capsule only	34.1	47.4	2.27
	" " "	3	" " "	34.8	48.6	2.28
	Aorta implanted intraperitoneally	2	Few Ca granules	35.7	47.7	2.18
	Agar mass implanted subcutaneously	1	" " "	33.2	46.2	2.27
	Aorta implanted subcutaneously	2	" " "	34.4	46.7	2.21
	Rabbit's own aorta		Few calcified plaques	32.8	51.8	2.59
	Rib			34.8	55.6	2.61
	Femur			36.4	54.9	2.46

Control rabbits given no viosterol

253	Agar mass implanted intraperitoneally	3	Granules in agar	34.4	48.1	2.29
	Aorta implanted intraperitoneally	3	No gross calcification	30.2	44.7	2.42
	Aorta implanted subcutaneously	3	Calcification doubtful	36.6	44.7	1.99
254	" intraperitoneally	3	Firm at one point	32.3	46.2	2.28
255	No calcified material					
256	Agar mass implanted intraperitoneally	3	Ca in capsule only	37.3	51.6	2.26

evidence of calcification was found in it.   In the three animals
surviving for the duration of the experiment a satisfactory increase
in blood calcium was noted and small granular calcium deposits
were found in various locations, as indicated in Table III.

Four control rabbits, which received no viosterol, were operated
on once.   A single piece of agar gel and a piece of boiled aorta
were introduced both intraperitoneally and subcutaneously.   The
control animals were killed 3 months after the operation and an
occasional granule of calcium-containing material was present, as
noted in Table III.

The calcific material from the rabbits was ashed with KOH
and analyzed as described above.   Table III is a summary of the
findings.

DISCUSSION

Wells observed that the inorganic composition of pathological
calcific deposits tends to approximate that of adult bone; i.e., an
average of 52 per cent of Ca as CaO, 40 per cent of P as P_2O_5, and
0.7 per cent of Mg as MgO.   The figures of Barillé (1), who
picked out calcific deposits from two aortas, are in substantial
agreement.   Schoenheimer (2) ashed bone and sclerotic material
from two aortas according to Gabriel's glycerol-KOH method, and
obtained somewhat lower results.   Kramer and Shear (7) ana-
lyzed normal bone and specimens of pathological calcification, and
found that the ratio of residual calcium to residual phosphorus
averaged 1.96, except in three calcified uterine fibromyomata,
where the ratio was 2.2.   We are unable to calculate residual
figures, as CO_2 determinations were not feasible on the small
amounts of material available in most instances.   However, the
results of the calcium and phosphorus determinations described
above are in the same range as those previously reported.   An
unexpected finding, however, is the unusually high content of
magnesium in the vascular plaques, approximately 0.9 per cent
calculated as MgO.   (Control analyses of four adult human bones
by our method averaged 0.75 per cent MgO.)

It may be emphasized that the composition of calcified deposits
is similar whether from human or rabbit sources.   Furthermore,
the granular material which was undoubtedly in the process of
deposition in the rabbits has the same inorganic composition as

the probably older, more static, vascular plaques. This is in contrast to the observation of Kramer and Shear (7) that the ash of growing rat bone has a higher residual Ca:P ratio than adult bone. This similarity of composition probably merely reflects the fact, emphasized by Wells, that with the blood continually passing between the bones and calcified areas the composition of the two must inevitably become similar.

The degree of calcification of colloidal gels *in vivo* was somewhat disappointing. Even the presence of well preserved elastic fibers in the pieces of aorta implanted did not suffice to attract an abundance of calcium salts. Calcium salts were laid down in some of the agar masses, but in most cases the deposition took the form of thin confluent plaques in the connective tissue capsule which formed about the agar. The agar implanted subcutaneously tended to disintegrate *in situ*. In only one such mass were calcium granules definitely present. Perhaps a longer residence of the foreign bodies within the animals would have led to more extensive deposits. The administration of toxic doses of viosterol increased markedly the amount of calcification. An increased ash content of the kidneys of rats given toxic doses of viosterol was found by Light, Miller, and Frey (8) and by Morgan, Kimmel, Thomas, and Samisch (9, 10), but gross calcification was not observed.

SUMMARY

1. The inorganic composition of calcified deposits in atherosclerosis of the aorta and in Mönckeberg's sclerosis of large arteries is similar to that of adult bone. The average composition of such deposits is: CaO 49.5 per cent, P_2O_5 36.1 per cent, MgO 0.90 per cent, and Ca : P 2.24.

2. Agar gel and sterile aortic tissue implanted intraperitoneally and subcutaneously into rabbits undergo a small amount of calcification. Administration of toxic doses of viosterol to the rabbits increases calcification of implanted colloids markedly. The average composition of these calcific deposits is: CaO 46.8 per cent, P_2O_5 32.7 per cent, and Ca:P 2.34.

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THE EFFECT OF DEXTROSE INGESTION ON THE CHOLESTEROL FRACTIONS OF THE BLOOD*

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Previous reports from this laboratory have indicated that the ingestion of dextrose in human subjects is associated with an increase, decrease, or no change in the total cholesterol content of the blood (1). The assumption was made that these inconstant changes in the blood cholesterol were in some way due to the metabolic effects of this sugar, since an increased concentration of dextrose in the blood, *per se*, exerting purely physical influences, would tend to diminish the blood cholesterol. This latter conclusion was drawn from experiments in which urea was fed (2). For reasons to be discussed later, it seemed important to pursue further the relation between cholesterol and dextrose in the blood; in the present communication, the alterations in the total, free, and ester cholesterol after dextrose ingestion are discussed.

Material and Methods

This report is based on studies of twenty-eight subjects, all adults, who had previously fasted for about 14 hours. Eight persons served as a control group; the variations of the cholesterol fractions were determined over a period of 1 hour. Each of the remaining twenty subjects was given a total of 100 gm. of dextrose in approximately 15 per cent solution by mouth in two equally divided doses ½ hour apart. The whole blood sugar and the serum cholesterol fractions were obtained before, ½ hour after the first dose of dextrose, and, again, ½ hour after the second dose.

The whole blood sugar was determined by the method of Folin and Wu (3); the cholesterol fractions, according to the procedure of Schoenheimer and Sperry (4) as modified for the colorimeter by Fitz (5).

TABLE 1

Variations of Cholesterol Fractions in Serum over a Period of 1 Hour in Eight Fasting Subjects

Subject No.	Specimen	Total cholesterol	Free cholesterol	Ester cholesterol	Ratio, ester to free cholesterol	Deviation
	min.	*mg. per cent*	*mg. per cent*	*mg. per cent*		*per cent*
1	0	200	51	149	2 92	
	30	198	53	145	2.74	
	60	199	54	145	2.69	−7
2	0	323	87	236	2 71	
	30	325	84	241	2 87	
	60	328	84	244	2.90	+6
3	0	183	61	122	2.00	
	30	185	61	124	2 03	
	60	183	63	120	1 90	−2
4	0	238	71	167	2 35	
	30	238	71	167	2 35	
	60	238	69	169	2 45	+2
5	0	179	59	120	2.04	
	30	180	60	120	2 00	
	60	186	59	127	2.15	+2
6	0	227	66	161	2.44	
	30	227	64	163	2.55	
	60	228	65	163	2.51	+4
7	0	208	68	140	2.06	
	30	214	66	148	2 24	
	60	209	67	142	2.12	+6
8	0	182	45	137	3.04	
	30					
	60	182	46	136	3.00	−1

Results

The results obtained in a control group of eight fasting subjects are shown in Table I.   The calculations of the ratio of ester to free cholesterol and in each case the percentage deviation of the mean of the 30 minute and 60 minute ratios of ester to free cholesterol from the initial reading are included in this and in the subsequent

TABLE II

*Effect of Ingestion of Two Doses of 50 Gm. of Dextrose Each Given ½ Hour
Apart on Cholesterol Partition of Serum in Twenty Fasting Subjects*

Subject No.	Specimen	Sugar	Total cholesterol	Free cholesterol	Ester cholesterol	Ratio, ester to free cholesterol	Deviation
		min.	*mg. per cent*	*mg. per cent*	*mg. per cent*		*per cent*
9	Control	75	136	49	87	1.78	
	30	92	151	45	106	2.33	
	60	106	144	46	98	2.16	+27
10	Control	96	171	66	105	1.60	
	30	158	319	94	225	2 41	
	60	188	162	62	100	1.63	+26
11	Control	105	169	64	105	1.65	
	30	169	305	102	203	1.99	
	60	208	296	89	207	2.34	+31
12	Control	72	198	73	125	1.72	
	30	135	207	75	132	1.75	
	60	201	222	77	145	1.89	+6
13	Control	192	222	71	151	2.11	
	30	259	226	72	154	2.15	
	60	349	221	76	145	1.90	−4
14	Control	92	214	75	139	1.85	
	30	150	234	73	161	2.19	
	60	188	223	72	151	2.09	+16
15	Control	97	221	78	143	1.82	
	30	107	247	80	167	2.11	
	60	139	257	85	172	2.02	+13
16	Control	91	166	63	103	1.63	
	30	136	173	57	116	2.03	
	60	172	172	56	116	2.05	+25
17	Control	94	268	87	181	2.10	
	30	111	277	86	191	2.22	
	60	135	277	82	195	2.37	+9
18	Control	84	171	57	114	2.02	
	30	101	180	62	118	1.90	
	60	144	187	63	124	1.97	−4
19	Control	91	277	93	184	1.99	
	30	139	284	92	192	2.10	
	60	179	297	94	203	2.16	+7
20	Control	87	186	62	124	2.01	
	30	135	192	60	132	2.23	
	60	172	186	57	129	2.27	+13
21	Control	97	259	82	177	2.14	
	30	136	256	85	171	2.01	
	60	174	255	78	177	2.27	0

TABLE II—*Concluded*

Subject No.	Specimen	Sugar	Total cholesterol	Free cholesterol	Ester cholesterol	Ratio, ester to free cholesterol	Deviation
		min.	*mg per cent*	*mg per cent*	*mg. per cent*		*per cent*
22	Control	88	331	97	234	2 41	
	30	109	330	98	232	2.37	
	60	192	307	78	229	2.93	+10
23	Control	96	215	74	141	1.90	
	30	125	215	76	139	1.84	
	60	158	267	75	192	2.56	+16
24	Control	83	281	95	186	1.95	
	30	147	307	94	213	2 26	
	60	224	308	104	204	1.96	+8
25	Control	89	185	59	126	2.12	
	30	134	230	59	171	2.87	
	60	136	186	50	131	2.67	+30
26	Control	103	268	78	190	2.47	
	30	150	257	74	183	2.46	
	60	160	261	80	181	2.26	−5
27	Control	106	244	74	170	2.29	
	30	146	242	82	160	1.95	
	60	155	235	74	161	2.19	−10
28	Control	89	158	58	100	1 76	
	30	135	172	61	111	1 82	
	60	143	174	60	114	1.89	+7

protocol. (In Table II, the initial determination represents the control period before the ingestion of dextrose.) The constancy of the serum cholesterol fractions in fasting subjects over a period of 1 hour (three determinations) is obvious from Table I and needs no further comment. The ratios of ester to free cholesterol varied from +6 per cent to −7 per cent.

Table II shows the effect of the ingestion of two doses of 50 gm. of dextrose, each given ½ hour apart, on the serum cholesterol fractions in twenty fasting subjects. In twelve of the twenty cases (60 per cent), the total cholesterol in the serum showed a definite increase after dextrose ingestion (Cases 9 to 12, 14, 15, 18, 19, 23 to 25, and 28); no change or a slight decrease in the total serum cholesterol was observed in the remainder (eight cases). The increase in the total cholesterol was invariably due to an augmentation in the ester cholesterol fraction, the free cholesterol

remaining remarkably constant. Cases 10, 11, and 22 were exceptional. In the first two instances the free cholesterol showed a significant rise following dextrose ingestion, and in the last case a definite fall in the 60 minute period. The increase of the ester cholesterol fraction in the serum and the relative constancy of the free cholesterol after the administration of dextrose are indicated by the rather marked positive per cent deviation of the ratio of ester to free cholesterol; in seven instances (Cases 9 to 11, 14, 16, 23, and 25), the ratios showed an increase of over 15 per cent.

DISCUSSION

The present study was undertaken in an attempt to throw some light on the association between the blood cholesterol and carbohydrate metabolism; that such a relationship probably existed was assumed in a previous report from this laboratory (1). A number of workers have shown that the starvation hypercholesterolemia in animal and man could be reduced by the administration of dextrose. Rony and Ching (6) concluded from their experiments on the alimentary lipemia in dogs that carbohydrate metabolism plays an important if not an essential rôle in the regulation of the blood fat level of normal dogs; the thesis was presented that glycogen formation favors fat deposition. In a recent communication, Petersilie (7) formulated an interesting hypothesis linking carbohydrate, fat, and cholesterol metabolism.

In this paper, we have shown that the ingestion of dextrose by man is often accompanied by an increase in the total blood cholesterol; when this increase occurs, it is invariably due to an augmentation of the ester cholesterol fraction, the free cholesterol remaining remarkably constant. If it is correct to assume that an increment of cholesterol esters in the blood represents an increase in the amount of fat transported to and from the tissues (Bloor (8)), the conclusion reached by Rony and Ching, that dextrose ingestion and the resultant formation of glycogen augment fat transport and favor its deposition in the tissues, appears substantiated from the results presented in this paper. It may also be assumed from the recent work of Nagao (9) and of Abrami et al. (10) concerning the antagonistic processes of fat and glycogen deposition in the liver that the rise of cholesterol esters in the blood after the ingestion of dextrose represents the removal of liver fat incident to glycogen deposition.

SUMMARY

Twelve of twenty fasting subjects showed an appreciable increase in the total serum cholesterol after the ingestion of dextrose. When this increase in the total cholesterol occurred, it was invariably due to an increment in the ester cholesterol fraction, the free cholesterol remaining remarkably constant.   Some theoretical considerations concerning the relation between the function of cholesterol in the body and certain phases of carbohydrate metabolism are discussed.

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THE PURIFICATION AND PROPERTIES OF LYSOZYME

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Fleming (1) in 1922 reported the occurrence of a bacteriolytic principle, lysozyme, in egg white, tears, and other animal fluids. Since then much work has been devoted to its bacteriological behavior, but the only work of importance on its purification and chemical nature was that of Wolff (2). Wolff precipitated diluted egg white with colloidal iron, evaporated the filtrate to a small volume, precipitated the lysozyme with acetone, and dialyzed the aqueous solution of the precipitate. The last two steps were repeated several times and finally a white powder was obtained, soluble in water, but insoluble in the common organic solvents. It was reported to give no Molisch reaction and to be free of sulfur, phosphorus, and nitrogen, although the biuret reaction was weakly positive.

In the present work an attempt was made to obtain the lysozyme from egg white in a purified form and to study its nature.

Preparation—The starting material for purification was a dry powder obtained by precipitation of native egg white with 9 volumes of ice-cold acetone. After the mixture had stood cold overnight, the precipitate was filtered off, washed well with acetone and ether, and dried in a heated vacuum desiccator over P_2O_5. Usually 14 to 15 per cent of the total weight of the egg white was thus obtained as a dry white powder with no loss of potency. Acetone precipitation at the original alkaline pH of egg white renders the greater part of the proteins insoluble, while the lysozyme retains its solubility in aqueous media.

In a procedure similar to that of Wolff, 40 gm. lots of the egg white powder were extracted with three 500 cc. portions of 0.9

per cent NaCl. To the combined extracts 120 cc. of commercial colloidal iron were added. If any colloidal iron remained in the solution after centrifuging, it was brought down with solid K_2HPO_4. The lysozyme in the supernatant liquid was then precipitated almost quantitatively by flavianic acid. However, the losses by adsorption on the iron precipitate were great, and the procedure was abandoned.

In the method finally adopted, 10 gm. lots of the egg white powder were extracted with 200 cc. of 50 per cent alcohol containing 10 per cent acetic acid at 60–70° for 20 to 30 minutes. These conditions were not injurious to the enzyme. The cooled mixture was filtered, and the filtrate evaporated under reduced pressure to a small volume. This was taken up in water and precipitated with 5 volumes of alcohol. The mixture stood cold overnight, and the precipitate was removed, washed with alcohol, taken up in 100 cc. of slightly alkaline water, and acidified with H_2SO_4 to maximal precipitation. The lysozyme was then precipitated from the supernatant solution by flavianic acid. The mixture stood cold for 24 to 48 hours, and the yellow precipitate was centrifuged off and washed abundantly with alcohol until the washings were colorless. The flavianate was in some instances dissolved in water made just pink to phenolphthalein with NaOH, then reprecipitated with dilute H_2SO_4 in the presence of a trace of flavianic acid. From the alcohol-wet precipitate the dye was removed by repeated extractions with 200 cc. portions of ice-cold 90 per cent alcohol containing 0.5 per cent NH_3.[1] The residue was washed free of ammonia with alcohol and ether and dried. The yield was 100 to 150 mg. from 10 gm. of egg white powder; the activity was 2000 to 6000 units per mg.[2]

[1] Flavianic acid was previously used successfully in the purification of hypophyseal hormones. Its removal by alcoholic ammonia was worked out at that time (3).

[2] The following procedure was employed in determining activity. Progressive dilutions (0.5 cc.) of the preparations in 0.9 per cent NaCl were mixed with 0.5 cc. of a saline suspension of the test organisms and incubated at 37°. Readings were usually made after 1 hour and 18 to 20 hours, the latter reading being regarded as more significant. The highest dilution where complete lysis occurred was taken as the end-point, although partial lysis was usually evident at much higher dilutions. The unit of activity was defined as the smallest amount of lysozyme causing complete

A further concentration was obtained by extraction with small amounts of water acidulated with acetic acid, followed either by reprecipitation with flavianic or picrolonic acid or by evaporation in a high vacuum over P_2O_5 while frozen. The flavianate or picrolonate could be further purified by recrystallization from 50 per cent aqueous pyridine. In this way, preparations of crystalline appearance have been obtained with an activity of 32,000 units per mg. Efforts to devise a standard procedure for crystallization met with failure.   ·

The principal contaminant of the different lysozyme preparations was an egg mucoid; the mucoid, however, was not precipitated by flavianic acid, and could be easily obtained from the flavianic acid supernatant solution by alcohol precipitation. Such a precipitate contained about 11 per cent nitrogen and 25 per cent reducing sugar after hydrolysis, and was almost devoid of any lysozyme activity.

Properties—The purest lysozyme preparations were basic in nature, being soluble only in acidified aqueous media[3] and insoluble in pure organic solvents. They contained about 15 per cent nitrogen, a small amount of sulfur present as sulfhydryl, and a small amount of phosphorus. A highly purified preparation which appeared crystalline under the polarizing microscope had the following composition (in per cent): C 48.65, H 6.44, N 15.33, ash 3.31, P 0.25, S 0.64.[4] With phosphorus and sulfur as a basis, the minimum molecular weight is about 25,000.

The biuret, glyoxylic acid, Greenberg phenol (5), and nitroprusside reactions were positive, and the Molisch negative. Bromine in glacial acetic acid was readily decolorized. Lysozyme solutions were not precipitated by trichloroacetic or sulfosalicylic acids and only incompletely by tungstic acid. Perchloric acid

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lysis in the test. The test organism was a typical *Sarcina* isolated from the air of the laboratory. 18 hour cultures on agar plates or slants were washed off with saline, and the suspension diluted to the density of $BaSO_4$ standard No. 8 (4).

[3] This fact is in accordance with the behavior upon filtration through Berkefeld filters: in alkaline media, no lysozyme passes through; in media acidified with acetic acid, only part of it passes; in dilute mineral acid, it is entirely filtrable.

[4] We thank Professor H. T. Clarke of the Department of Biological Chemistry for the microanalyses made in his laboratory.

precipitated the enzyme. Some salts of heavy metals (as gold and silver) precipitated lysozyme with simultaneous inactivation.

Lysozyme is very stable toward heat and acid (6). Solutions in 2 per cent acetic acid were kept at 100° for 45 minutes with no loss of lytic activity. Neutral preparations treated in this way lost all activity. At pH 9, heating for 5 minutes at 100° destroyed most of the activity. At room temperature, treatment with 0.01 N NaOH for 10 minutes lowered the activity from 3000 to 80 units per mg.

TABLE I

Reversible Inactivation of Lysozyme by Iodine and Cuprous Oxide; Reactivation by Hydrogen Sulfide

Mixture A. 2 cc. lysozyme solution + 0.3 cc. saline
" B. 4 " " " + 0.6 " 0.01 N I₂ in KI for 7 minutes
" C. 2 " Mixture B saturated with H₂S
" D. 4 " lysozyme solution + 0.6 cc. saline + 12 mg. Cu₂O for 60 minutes
" E. 2 " Mixture D saturated with H₂S

Mixtures D and E were centrifuged before being tested. Reading made after 1 hour at 37°.

4 indicates complete clearing; 0 indicates no clearing. A control with saline saturated with H₂S showed no lysis.

Mixture	Final dilution of mixtures, 1 to							
	20	40	80	160	320	640	1280	2560
A	4	4	4	3	2	1	0	0
B	1	0	0	0	0	0	0	0
C	4	3	1	1	0	0	0	0
D	4	3	2	1	1	1	0	0
E	4	4	4	4	3	2	1	1

The destructive action of peroxides was observed during work with an ordinary vacuum-distilled sample of dioxane as a precipitant; the activity was completely destroyed. After the removal of peroxides (7), the dioxane had no deleterious effect.

The sensitivity of lysozyme to alkali and peroxide suggested the necessity of an intact sulfhydryl group in the molecule. Furthermore, iodoacetic acid inactivated the enzyme. Sozoiodolic acid, used by Ackermann (8) for the crystallization of amines, gave in acid solution a complete precipitation of the ferment; a solution of the precipitate was inactive. Precipitation and inactivation

were also obtained when lysozyme solutions were iodized with iodine in potassium iodide. Cuprous oxide, which reacts with sulfhydryl groups to form mercaptides (9), decreased the activity of lysozyme. The inactivation by iodine or cuprous oxide was at least partially reversible, as Tables I and II indicate.

In Table I hydrogen sulfide was the reactivating agent; in Table II, sulfite. The latter experiment further differed in that the only variant throughout the tubes of a given series was the lysozyme concentration. In the second experiment, after incu-

TABLE II

Reversible Inactivation of Lysozyme by Iodine; Reactivation by Sulfite

Each series contained 0.25 cc. of progressive dilutions of lysozyme with the following additions.

Addition A. 0.25 cc. 2.5% KI + 0.25 cc. saline + 0.75 cc. test organisms

• "   B. 0.25 " 0.1 N I$_2$ in 2.5% KI + 0.25 cc. saline + 0.75 cc. test organisms

"   C. 0.25 cc. 0.1 N I$_2$ in 2.5% KI + 0.25 cc. 2% Na$_2$SO$_3$ (neutralized with HCl) + 0.75 cc. test organisms

In Mixtures B and C, the I$_2$ solution was allowed to act for 10 minutes before further additions were made. Reading made after 1 hour at 37°.

4 indicates complete clearing; 0 indicates no lysis. A control of 0.50 cc. of saline + 0.25 cc. of sulfite + 0.75 cc. of test organisms showed no lysis.

Mixture	Final dilution of lysozyme solution, 1 to									
	6	12	24	48	96	192	384	768	1536	3072
A	4	4	4	4	4	4	4	4	4	3
B	0	0	0	0	0	0	0	0	0	0
C	4	3	2	1	1	1	1	1	1	1

bation 1 drop of 40 per cent NaOH was added to each tube to clear up the turbidity imparted to the lysozyme solution by the more concentrated iodine; the alkali had no effect on the appearance of the organisms alone. Hydrogen cyanide was also found partially to·reactivate iodine-inactivated preparations.[5]

DISCUSSION

With the process of purification described a substance was obtained with the properties of a basic polypeptide having 15.3 per

[5] Attempts to attack otherwise non-susceptible bacteria with lysozyme activated with HCN have been unsuccessful.

cent nitrogen. Wolff (2) claimed that lysozyme was nitrogen-free, but gave a weak biuret reaction. His purification method consisted in the removal of impurities with colloidal iron and precipitation by acetone. In our experience the use of colloidal iron caused no change in the nitrogen content of the product. Wolff's preparations, like ours, gave no Molisch reaction and no reducing sugar after hydrolysis. In his first paper (2) he stated that lysozyme was destroyed by tryptic digestion, but subsequently he reported that a 3 hour incubation with trypsin did not destroy it. It is difficult to conceive a nitrogen-free, non-sugar neutral substance of high molecular weight, soluble in aqueous solutions only and insoluble in all organic solvents. Wolff stated, however, that lysozyme was extracted from aqueous solutions by vegetable oils and that it was very difficult to remove the lysozyme from the oil. On repeating this, we found lysozyme in the interphase, but it could easily be reextracted with a concentrated NaCl solution after dilution with petroleum ether.

It may be of interest to compare the potency of our preparations with those of Wolff. The latter used a different organism (*Micrococcus lysodeikticus*) and a different incubation temperature (56°) which he found to be the optimum. Native egg white had in Wolff's test a potency about 40 times greater than in our test. Allowing for this factor and calculating in our terms, his best preparations had an activity of 20,000, 6,400, and 11,300 units per mg.; that is, about the same as our purest, 16,000 to 32,000.

According to Shwachman, Hellerman, and Cohen (10), pneumococcus hemolysin can be reversibly oxidized and reduced, the oxidized form being inactive. We found the same to be true for lysozyme. This suggests the possible rôle of peroxide in many bacteria as a defense against lytic agents. It may also throw light on the decreased resistance to infection in avitaminosis A in which it seems to be difficult for the body to maintain substances in the reduced form.[6]

SUMMARY

The preparation of lysozyme from acetone-dried egg white was studied. Lysozyme can best be freed from the tenaciously ad-

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[6] It has been shown that the lysozyme content is greatly diminished in the tears of experimental animals (11) and human patients (12) deficient in vitamin A; the keratomalacia disappears following local treatment with lysozyme.

hering mucoid in the extracts by precipitation with flavianic acid. Lysozyme is apparently a basic polypeptide, having a nitrogen content of 15.3 per cent and giving a number of protein reactions. From the presence of sulfhydryl, its inactivation by alkali, peroxide, iodine, and cuprous oxide, and its reactivation by hydrogen sulfide, sulfite, and hydrogen cyanide, it is concluded that lysozyme acts only in the reduced state.

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STUDIES ON ANIMAL LIPIDS*

X. THE NATURE OF CEREBROSIDASE. ITS RELATION TO THE SPLITTING OF POLYDIAMINOPHOSPHATIDE BY POLYDIAMINOPHOSPHATASE†

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. The presence of a cerebrosidase in tissues has not been demonstrated. Rosenheim (1) and Jungmann and Kimmelstiel (2) attempted by enzymatic action to break down the cerebrosides, but their results were unsatisfactory.

Rosenheim found that the lipase of the pancreas and emulsin would not split cerebron. Jungmann and Kimmelstiel believed that they demonstrated a slight splitting of galactose of cerebrosides within the brain tissue after death. We have confirmed the negative reports of Rosenheim. True cerebrosidase is practically inactive. For activity cerebrosidase requires an activator. Compounds with an —SH radical, as H_2S, cysteine, —SH glutathione, or other reversible systems such as ascorbic acid may be employed as activators.

Knowledge concerning the importance of reversible systems in enzymatic processes dates from the discovery of glutathione by Hopkins (3). Waldschmidt-Leitz and coworkers (4) and Grassmann and associates (5) showed that the reduced glutathione is the natural activator of certain intracellular enzymes. This included enzymes originating in animals as well as in plants. Later it was found that vitamin C has a similar function (6).

We know now that the efficacy of such reversible systems is

due to their influence on the oxygen potential in the cell (7). Our experiments concerning the activation of cerebrosidase by the —SH groups (Tables II and III) indicate that enzymatic changes in lipids are associated with similar reduction systems, as in the case of proteins and carbohydrates.

The basic substances of diaminophosphatides and cerebrosides are ceramides, such as lignocerylsphingosine.

Ceramide
Lignocerylsphingosine
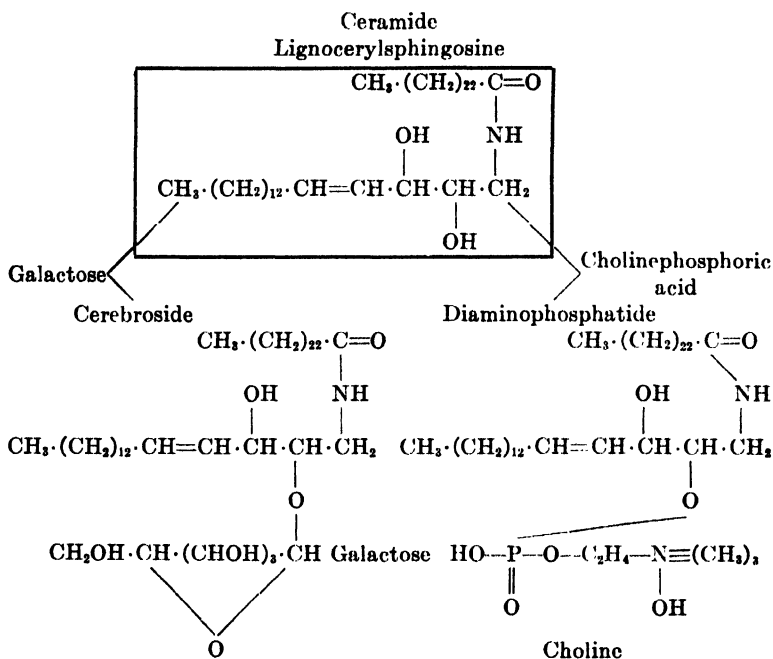
Thannhauser, Fränkel, and Bielschovsky (8) and Tropp (9) have demonstrated the presence of lignocerylsphingosine in liver, lung, and spleen. One may assume that the esterification of lignocerylsphingosine with cholinephosphoric acid results in a diaminophosphatide; on the other hand, the glycoside linkage of galactose with the ceramide produces a cerebroside. Therefore, we were led to study the relationship of the polydiaminophosphatase, which our former coworker, Rossi (10), has described, to the activity of cerebrosidase.

It seems possible that in the anabolism and catabolism of the

above substances there exists a reciprocal relationship between diaminophosphatase and cerebrosidase. Waldschmidt-Leitz and coworkers (11) have found that the —SH group retards the activity of phosphatases from various sources, such as kidney and yeast. We were able to observe the same relationship when cysteine depressed the activity of the polydiaminophosphatase (Table IV).

The same system which activates the cerebrosidase serves to retard, to a certain degree, the activity of the enzyme which splits off the phosphoric acid.

The results mentioned indicate that the —SH radical is especially significant in the metabolism of nerve cells, more particularly in its reciprocal action on the oxidation-reduction potential of the cells. We hope that further experiments which are now in process may throw more light on this subject.

The processes concerning the polydiaminophosphatase and cerebrosidase described above may give the clinician a hint as to the pathogenesis of Gaucher's disease on the one hand and Niemann-Pick's disease and the amaurotic idiocy on the other

In Gaucher's disease the cerebrosides are increased and stored in the reticuloendothelial cells. In Niemann-Pick's disease (12), and very likely in the amaurotic idiocy, there is an increase and deposition of the diaminophosphatides. It is possible that these rare diseases are caused by a disturbance in the equilibrium between cerebrosidase and diaminophosphatase.

EXPERIMENTAL

The determination of galactose is carried out according to the Hagedorn-Jensen semi-micromethod as modified by Hanes (13). The galactose equivalents as compared with those of glucose are given in Table I.

Preparation of Substrate—0.500 gm. of cerebron is dissolved in 5 cc. of alcohol with heat and introduced with a pipette into water at 80–90°. The alcohol is completely driven out of the colloidal solution by repeated evaporation and addition of distilled water to its former volume. Finally the solution, free from alcohol, is diluted with distilled water to bring the concentration to about 0.1 per cent.

The enzyme solution is prepared from both organs according to

the method of Klein (14).   3 gm. of pulverized organ are extracted with 100 cc. of physiological sodium chloride solution by shaking for 5 hours.   This is followed by dialysis for 48 hours by running water.

Cerebrosidase

Influence of H_2S, Cysteine, Ascorbic Acid, and —SH Glutathione on Activity of Cerebrosidase—To 1 cc. of dialyzed enzyme solution are added 20 mg. of cysteine HCl, previously neutralized, 20 mg. of *l*-ascorbic acid, 20 mg. of —SH glutathione, or a stream of H_2S for 1 hour.   The other samples are incubated for 60 minutes (pH 7) at 30°.

TABLE I

Comparison of Glucose and Galactose Equivalents

Sugar	0.01 N $Na_2S_2O_3$ (glucose)	0.01 N $Na_2S_2O_3$ (galactose)
mg.	*cc.*	*cc.*
0.20	0.58	0.46
0.50	1.45	1.15
0.80	2.32	1.85
1.00	2.91	2.31
1.50	4.40	3.50
2.00	5.80	4.61
2.50	7.38	5.87
3.00	8.93	7.10

After incubation, 3 cc. of veronal buffer (Michaelis (15)) and 3 cc. or 6 cc. of the substrate solution (2.31 or 4.61 mg. of cerebron) are added.   The total volume of 15 cc. is incubated for 16 hours at 37°.   Results for this mixture are given in Table II.   2.31 cc. of 0.01 N $Na_2S_2O_3$ would indicate the complete breakdown of the cerebroside.

At the end of this period the H_2S is driven off by passing CO_2 through for 30 minutes or by heating the solution on a steam bath for the same time.

Pancreas-Glycerol Solution and Emulsin—To either 0.2 cc. of pancreas-glycerol solution (1:10) or 5 mg. of emulsin are added 20 mg. of cysteine HCl, previously neutralized.   The mixture is incubated for 60 minutes at 30°, followed by addition of 3 cc. of

veronal buffer and 6 cc. of substrate solution. The total volume of 15 cc. is kept for 16 hours at 37°.

The above minimal splitting of cerebron obtained with the original enzyme solution may result from the method of prepara-

TABLE II

Activation of Cerebrosidase by —SH Groups

Time, 16 hours. The results are measured in cc.

Organ	pH	Without activator, 6 cc.	H₂S		Cysteine, 6 cc.	l-Ascorbic acid, 6 cc.	—SH gluta- thione, 6 cc.
			3 cc.	6 cc.			
Brain.............	4.9	0.08		1.89	1.95	1.51	1.94
"	7.0	0.04		1.96	1.98	1.58	1.99
"	8.9			0.46	0.51	0.15	0.63
Spleen...........	4.9	0.15	1.19	2.30	2.03	1.74	2.14
• "	7.0	0.23	1.13	2.24	2.09	1.65	2.20
"	8.9	0.11	0.56	0.70	0.68	0.35	0.81
Pancreas..........	4.9	0.09			1.70		
"	7.0	0.13			1.54		
"	8.9	0.18			0.37		
Emulsin..........	4.9	0.09			0.65		
"	7.0	0.08			1.38		
"	8.9	0.08			0.28		

TABLE III

Activation of Cerebrosidase in Presence of Iodoacetic Acid

Time, 16 hours. The results are measured in cc.

Organ	pH	Without activator	Cysteine	Cysteine + iodoacetic acid
Brain .	4.9	0.10	1.84	0.03
	7.0	0.07	1.90	
	8.9	0.02	0.60	
Spleen.	4.9	0.17	2.03	0.13
	7.0	0.17	1.95	0 20
	8.9	0.12	0.60	0.05

tion. Another experiment was performed to prove the reduction system responsible for this specific activation (see Table III). It is known (16) that iodoacetic acid retards the activators used in our experiments. The influence of iodoacetic acid in a solution

containing cysteine was investigated as an example. The conditions of the experiment are the same as given in Table II. The only difference is the addition of 20 mg. of iodoacetic acid to the system.

These experiments demonstrate that cerebrosidase needs as activators compounds with an —SH radical. Similar activation of cerebrosidase may occur also during the autolysis of a tissue. According to Waldschmidt-Leitz (17), —SH glutathione is formed in the autolysis of a tissue as an effect of a lower oxygen potential. In the same way the splitting of small amounts of cerebrosides in brain cysts (18) may be illustrated.

TABLE IV

Influence of Cysteine on Polydiaminophosphatase

Organ	Time	pH	Original solution	Cysteine		Cysteine + iodoacetic acid	
	hrs.		*mg. P*	*mg. P*	*per cent deactivation*	*mg. P*	*per cent activity*
Spleen...............	72	4.9	0 081	0.052	35 8		
"　　...............	72	7.0	0 085	0 034	60		
"　　...............	72	7.9	0.197	0 089	55		
"　　...............	48	7.9	0.135	0 081	40	0.113	83.7
Brain...............	72	4.9	0 083	0 062	25.3		
"　　...............	72	7 0	0 097	0.063	35 1		
"　　...............	72	7.9	0.188	0.066	65		
"　　...............	48	7 9	0.122	0 058	52 5	0 094	77

Influence of Cysteine on Polydiaminophosphatase—To 3 cc. of dialyzed enzyme solution are added 20 mg. of cysteine HCl, neutralized, or 20 mg. of cysteine HCl and 20 mg. of iodoacetic acid incubated for 60 minutes at 30°, followed by 3 cc. of veronal buffer, 2 cc. of 0.1 M $MgSO_4$, and 5 cc. of substrate solution (10), equivalent to 0.360 mg. of P. The total volume of 15 cc. was kept for 72 hours at 37°. The determination of phosphorus was carried out according to the method of Briggs (19). The results are given in Table IV for the whole mixture in mg. of phosphorus.

SUMMARY

1. Cerebrosidase is practically inactive.

2. The activators are H_2S, cysteine, —SH glutathione, and *l*-ascorbic acid.

3. Activators which stimulate cerebrosidase activity depress the activity of polydiaminophosphatase.

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THE ISOLATION FROM WHEAT GERM OIL OF AN ALCOHOL, α-TOCOPHEROL, HAVING THE PROPERTIES OF VITAMIN E*

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The first attempt to concentrate vitamin E was made by Evans and Burr in 1927 (1). The non-saponifiable matter from wheat germ oil was treated successively with pentane and methanol, from which the bulk of the sterols and some oily material separated. By distribution between pentane and 92 per cent methanol the xanthophyll pigments and some other inactive material were removed. There was thus obtained a red oil which in a single dose of 10 mg. enabled the test rats to bear litters. Further concentration was secured by high vacuum distillation, but this step was accompanied by considerable loss.

Olcott (2) and Olcott and Mattill (3, 4) also prepared concentrates from wheat germ oil, cottonseed oil, and lettuce by procedures analogous to those of Evans and Burr, except that in their hands vacuum distillation was a much more effective tool. Concentrates potent in a single dose of 3 mg. were secured. A very

potent concentrate was obtained by Drummond, Singer, and MacWalter (5) by fractionally adsorbing the sterol-free non-saponifiable fraction of wheat germ oil on a column of Brockmann's alumina.

Spectroscopic studies by Martin, Moore, Schmidt, and Bowden (6), Olcott (2, 7), and Drummond, Singer, and MacWalter showed that vitamin concentrates from cottonseed and wheat germ oils possessed a strong absorption band with a maximum at 2940 Å. Martin, Moore, Schmidt, and Bowden and Drummond, Singer, and MacWalter expressed the belief that this band is probably due to the vitamin. Drummond's concentrates showed considerable parallelism between the intensity of the absorption and the vitamin potency; the most potent preparations showed maximal absorption at 2940 Å., $E_{1\ cm.}^{1\ per\ cent} = 54$. However, Olcott (8) has concluded that the substance showing this absorption is not the vitamin. He based this conclusion upon four lines of evidence; viz., (1) he obtained a fraction from palm oil showing intense absorption here but with little or no vitamin activity; (2) acetylation caused a shift in the absorption maximum of both the wheat germ oil and palm oil concentrates to 2810 Å., but it did not interfere with the vitamin E potency of the wheat germ oil concentrate; (3) the concentrate from lettuce, although quite potent, showed no band at 2940 Å.; (4) by treating a cottonseed oil concentrate with methyl alcoholic silver nitrate, it was possible to destroy completely the absorption at 2940 Å. and still retain a considerable proportion of the vitamin activity.

Olcott (8) has shown that the vitamin is an alcohol, since it is inactivated by phenyl isocyanate, and the activity can subsequently be restored by hydrolysis with dilute alkali. By conversion to the methyl ether the vitamin activity was completely destroyed.

It is hardly necessary to state that if much progress is to be made in the elucidation of the structure of vitamin E, it must be isolated in pure form. Our plan of attack was to find a solid derivative of the vitamin which could be purified, and from which the vitamin could readily be regenerated. We first tried ketone reagents, but could find no indication of reaction, an observation which has been confirmed by Girard (quoted by Drummond, Singer, and MacWalter (5)). Next we tried a number of alcohol

reagents, but although most of these obviously reacted, they yielded only oily products. However, cyanic acid gave us three allophanates. One of these yielded an alcohol devoid of physiological activity. The other two were obviously related to the vitamin. The first of these melted at 158–160° after repeated recrystallization from ethyl or methyl alcohol or acetone. The analysis was in good agreement with the monoallophanate of an alcohol, $C_{29}H_{50}O_2$.[1] It separated as fine balls rather than definite
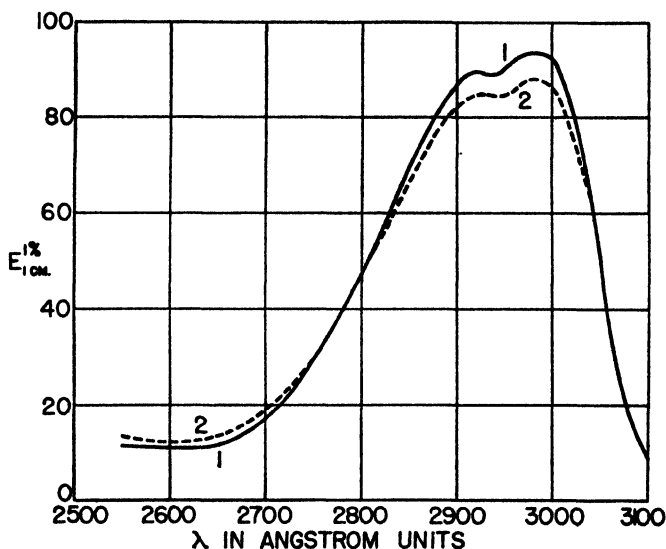
FIG. 1. Absorption spectrum of α-tocopherol in hexane. Curve 1, α-tocopherol from allophanate reconverted from p-nitrophenylurethane (Preparation 146-A); Curve 2, α-tocopherol from original allophanate.

crystals. On hydrolysis it yielded an oily alcohol; when this was fed in a single dose of 3 mg. litters were produced quite regularly, but only sporadically at the 1 mg. level. For this alcohol we propose the name "α-tocopherol."[2] The optical rotation of the allo-

[1] Analytical data secured by Drummond, Singer, and MacWalter (5) on their most potent preparation and its acetate are in excellent agreement with the value required by an alcohol, $C_{29}H_{50}O_2$, and its monoacetate.

[2] Tokos = childbirth; phero = to bear; -ol, indicating an alcohol. We wish to thank our colleague, Professor George M. Calhoun of the University of California, for the suggestion of this designation.

phanate in benzene or chloroform solution is very low, perhaps 0. The α-tocopherol shows strong absorption, with a maximum at 2980 Å., $E_{1\ cm.}^{1\ per\ cent} = 90 \pm 10$, and a secondary slightly less intense maximum at 2920 Å. (Fig. 1, Curve 2).

The second allophanate forms beautiful needles melting at 138°, and appears to be isomeric with α-tocopheryl allophanate, whose

TABLE I

Biological Activity of α-Tocopherol and Its Silver Nitrate Reaction Product

Prepara-tion No.	Level fed	Littering	Average No. per litter	Average weight of young per rat	Source of α-tocopherol
	mg.	*per cent*		*gm.*	
21	3	66	10	4.6	Allophanate as isolated
87-A	1	20	1	4.0	" " "
87-A	3	100	3.6	5.0	
71-A	3	75	7.6	5.6	" treated with bromine
87-B	1	0			" mixed with oil from
87-B	3	25	6	5.2	mother liquor of Preparation 87-A
90-B	2.5	100	10	5.2	Allophanate converted to *p*-nitro-phenylurethane
110-D	3	75	8.5	4 8	Allophanate fractionally adsorbed on CaCO₃; weakest adsorbed fraction
112-A	3	66	5.5	5 5	Allophanate adsorbed on CaCO₃; strongest adsorbed fraction
146-A	1	0			Original allophanate recrystal-
146-A	3	100	5.2	5.0	lized to melting point 158-160°, converted to *p*-nitrophenylure-thane, then reconverted to allo-phanate
71-B	4	50	5.5	4.4	Silver nitrate reaction product of α-tocopherol

solubility it closely parallels. However, the two are readily sepa-rated, since the crystalline allophanate separates from solution very much more slowly than that of α-tocopherol, particularly when it is impure. Biologically the alcohol from this allophanate is less potent than α-tocopherol. The absorption spectra of the two alco-hols appear very similar.

Attempts to fractionate α-tocopheryl allophanate by crystalliza-

tion from methyl or ethyl alcohol or acetone failed. To test its homogeneity, we adsorbed 106 mg. on a column of 100 gm. of calcium carbonate, developing the "chromatogram" with 1150 cc. of benzene. This washed 26 mg. of material through the column, which had the same melting point as the most strongly adsorbed fraction. The alcohols from the two end-fractions showed the same biological activity (Preparations 110-D and 112-A, Table I).

It was found that α-tocopherol reacted with p-nitrophenyl isocyanate to yield a nitrophenylurethane crystallizing in fine needles, which melted at 129–131°. The analysis of this substance was also in good agreement with the values required by a mono derivative of an alcohol, $C_{29}H_{50}O_2$, although this formula must be accepted with some reservation, since the difference in percentage composition between adjacent homologues is not great. The alcohol regenerated from the nitrophenylurethane, fed to four rats at a level of 2.5 mg., enabled all of them to bear good litters. The nitrophenylurethane was reconverted to the allophanate, and the product so obtained appeared microscopically identical with the original allophanate, and after a few recrystallizations, melted at 158–160°. α-Tocopherol from this reconverted allophanate showed the same physiological activity and absorption spectrum as the original product (Table I, Preparation 146-A; Fig. 1, Curve 1). Hence we feel justified in believing that we have obtained a homogeneous product which plays the biological rôle of vitamin E.

Olcott's finding that a concentrate from palm oil shows strong absorption at 2940 Å., with weak or no vitamin activity, is analogous to our experience with the alcohol from the 138° allophanate. His observation that treatment with methyl alcoholic silver nitrate caused the destruction of the absorption band at 2940 Å. with simultaneous persistence of vitamin activity was of great interest to us, although, as the reader will see, our interpretation of this phenomenon is different. We repeated the experiment with our pure substance—α-tocopherol. Blackening occurred as soon as the solution became warm[3] and after 5 minutes on the steam bath the

[3] There is a strange difference in the behavior of α-tocopherol and α-tocopheryl allophanate. When the allophanate is warmed with methyl alcoholic silver nitrate, no blackening takes place. The free alcohol readily decolorizes bromine, but the allophanate reacts extremely slowly. A sample of the allophanate was allowed to stand 1 hour in an excess of bromine in chloroform solution, and the alcohol regenerated from it showed both the

heating was discontinued. The absorption spectrum showed no sign of the band at 2920 to 2980 Å., but there were two new bands with maxima at 2710 and 2620 Å., having an intensity $E_{1\ cm.}^{1\ per\ cent}$ of 480 (Fig. 2). The reaction product was fed in a dose of 4 mg., and vitamin activity, although reduced, was not lost (Table I). If we assume that our α-tocopherol is a homogeneous (uncontaminated) substance, which seems justifiable, we are forced to the conclusion that the silver nitrate experiments have demonstrated that
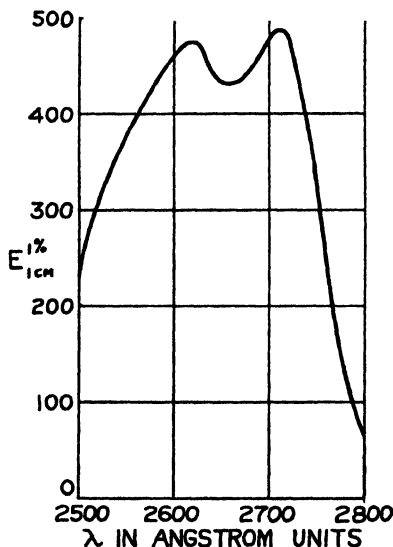
Fig. 2. Absorption spectrum of the reaction product of α-tocopherol with methyl alcoholic silver nitrate.

characteristic spectrum and normal vitamin activity (Table I, Preparation 71-A). A sample of the allophanate was allowed to stand in a weighed flask for 50 hours in an excess of bromine in chloroform. The chloroform and bromine were carefully removed, and there was no significant increase in weight. On standing 4 days with an excess of perbenzoic acid in chloroform solution, a sample of the allophanate produced no significant reduction of the perbenzoic acid. Were it not for the fact that the allophanate gives an intense color with tetranitromethane, one would imagine it to be a saturated body. Olcott and Mattill (4) noted that acetylated vitamin E concentrates are not destroyed by rancidity to which the free vitamin is extraordinarily sensitive.

vitamin E activity is not the property of a single molecular species; the situation in this respect thus resembles that in regard to vitamin D, to the precursors of vitamin A, and to the male and female sex hormones.

<center>EXPERIMENTAL</center>

Biological Assay—The procedure of Evans, Murphy, Archibald, and Cornish (9) was slightly modified. The diet of commercial casein 27.0, cooked corn-starch 35.0, fresh lard 22.0, dried brewers' yeast 10.0, Salt Mixture 185 (10) 4.0 was mixed and allowed to stand for 2 weeks at room temperature to allow the incipient rancidity of the lard to destroy any vitamin E present. Cod liver oil, 2 parts, was added just before feeding.

Rats 21 days old were placed on the diet, and were bred for their .trial gestation at 60 days of age, or as soon thereafter as their cycles permitted. The percentage of initial fertility under these conditions was very low. The test substances, dissolved in ethyl laurate, were administered by stomach tube to the rat under light ether anesthesia. Four animals were used for the test, and successful implantation was noted by finding the erythrocyte sign on the 13th day.

Measurement of the Absorption Spectra[4]—The absorption spectra were obtained with a small quartz spectrograph which photographed the region between 2000 and 5000 Å. on a 6 inch Eastman No. 33 plate, and had an average dispersion of 24 Å. per mm. between 2500 and 3000 Å. A hydrogen discharge tube was used as a continuous source of ultra-violet light. On each plate were recorded the absorption spectrum of the pure solvent (hexane, purified according to Twyman and Allsopp (11)) and that of the solution, several concentrations of the latter being used. With a cell 1.93 cm. long, a concentration of α-tocopherol of 0.005 per cent gave the most satisfactory range of blackening density. The blackening on the plate was determined with a .Zeiss recording microphotometer.

The calibration of the plates was made with screens of known transmission, as described by Harrison (12). Six calibration curves were made for the region between 2500 and 3000 Å., but as

these were found not to differ materially, an average curve was used for the interval.

By means of the calibration curves the intensity of the light was determined from the degree of blackening measured by the microphotometer, as described by Langstroth (13). Thus were measured the intensity of light passing through the cell filled with pure solvent, I_1, and the intensity of the light passing through the cell filled with solution, I_2.

The extinction coefficient, $E_{1\ cm.}^{1\ per\ cent}$, was calculated from the formula

$$E_{1\ cm.}^{1\ per\ cent} = \frac{1}{cL}\ \log_{10}\frac{I_1}{I_2}$$

where c equals the concentration of the solution in per cent and L the length of the cell in cm. For the curves of α-tocopherol, E was evaluated at intervals of every 10 Å. over the region of maximum absorption.

Preparation of Sterol-Free Non-Saponifiable Matter—The saponification of the wheat germ oil was conducted as described by Evans *et al.* (9) and the non-saponifiable fraction was extracted with ether rendered peroxide-free by distillation over stannous chloride. The non-saponifiable matter was taken up in petroleum ether, b.p. 85–110°, and allowed to stand overnight at 0° to permit the separation of the bulk of the sterols, which were filtered off and washed free of pigment. The petroleum ether solution was concentrated to contain about 10 per cent of solute and was washed several times with small volumes of 92 per cent methanol, which removed the xanthophylls and other inert material. The hydrocarbon solution, again concentrated to contain about 10 per cent solute, was extracted six times with an equal volume of dry methanol, saturated with petroleum ether, at 0°. This extracted the vitamin fairly completely, and was an effective means of removing certain oily substances difficultly soluble in methanol. The methanol solution was concentrated to small volume and allowed to stand at −18° to allow as much as possible of the remaining sterols to separate. The final trace of sterols was removed with digitonin from 90 per cent ethyl alcohol solution.

The non-saponifiable matter from 3.7 kilos of wheat germ oil was allowed to stand overnight at −18° in 250 cc. of methanol. For

the removal of the last trace of sterols, 12 gm. of digitonin were sufficient. There were thus obtained 21 gm. of sterol-free oil, potent in a 10 mg. dose.

Preparation of Allophanates—This was done essentially as described by Windaus, Gaede, Koeser, and Stein (14) for the irradiation products of ergosterol. The sterol-free oil, in 10 gm. portions, dissolved in 250 cc. of benzene, was saturated with cyanic acid gas generated by heating 15 gm. of cyanuric acid in a slow stream of CO_2. During the process the benzene solution was kept cool in an ice bath. The solution was allowed to stand a week at 5° for the reaction to go to completion. The cyamelide was filtered off and washed well with hot benzene, in which the desired allophanate was very soluble. The residue, on evaporation of the benzene, was dissolved in 10 volumes of methanol and allowed to stand overnight at 0°.

The oily precipitate was filtered off and the oil washed out with small amounts of petroleum ether, in which the desired allophanate was also, unfortunately, appreciably soluble. The allophanates were a mixture of a small amount of very sparingly soluble, high melting substance and a much larger amount of a much more soluble, lower melting product.

The cleanest separation was obtained by means of cold acetone, in which the low melting substance was moderately soluble and the high melting one very sparingly soluble.

The yield of high melting material from 3.7 kilos of wheat germ oil was about 100 mg., m.p. 250°.

Analysis—This analysis was made at Columbia University, through the kindness of Professor H. T. Clarke. The substance dried *in vacuo* at 80° without loss of weight.

C 74.77, 74.60; H 10.52, 10.30; N 5.39, 5.35
Calculated for $C_{32}H_{54}N_2O_5$.   C 74.65, H 10.58, N 5.44
"   " $C_{32}H_{52}N_2O_3$.   " 74.94, " 10.23, " 5.46

This may be the allophanate of β-amyrin, which Drummond, Singer, and MacWalter (5) found in their vitamin concentrate from wheat germ oil, and the formula of whose allophanate would be $C_{32}H_{52}N_2O_3$. The substance on hydrolysis yielded an alcohol having no indication of vitamin E potency, even in a dose of 10 mg. It was not further investigated.

Isolation of α-Tocopheryl Allophanate—The acetone filtrate from the high melting allophanate was evaporated to dryness *in vacuo* and taken up again in hot methanol, from which, on cooling, the α-tocopheryl allophanate separated, still contaminated with oil which was in part removed by careful washing with petroleum ether. Several recrystallizations and washing with petroleum ether were required to get the product completely free from oil, and several fractionations with acetone were required to remove completely the high melting allophanate. The pure α-tocopheryl allophanate melts at 158–160° and consists of matted granules about 0.01 mm. in diameter which are isotropic; mean index of refraction about 1.51.

The mother liquors from this allophanate, on concentration, have yielded small amounts of the crystalline 138° substance, but we have been unable to detect other solid allophanates.

Optical Rotation—56.2 mg. of substance in 3 cc. of $CHCl_3$ in a 1 dm. tube, with sodium D light, gave no measurable rotation.

Analysis—The following analytical data have been secured. The substance dried at 80° *in vacuo* gave no loss of weight.

	I	II	II	III	III	Mean
C	72.05	72.20	72.14	72.33	72.14	72.16
H	10.16	10.17	10.11	10.42	10.31	10.23
N	5.46			5.30	5.23	5.33

Calculated for $C_{31}H_{52}N_2O_4$. C 72.04, H 10.15, N 5.42
"   " $C_{32}H_{54}N_2O_4$. " 72.40, " 10.26, " 5.28

For I the analyst was Dr. Ing. A. Schoeller, Berlin; for II, Herrler, Leipsic; for III the analyses were made at Columbia University through the kindness of Professor H. T. Clarke.

The allophanate was hydrolyzed by refluxing ½ hour with 4 per cent methyl alcoholic potassium hydroxide in an atmosphere of hydrogen. The reaction mixture was cooled, 4 volumes of water were added, and the alcohol was extracted with peroxide-free ether. The alcohol was a light colored, viscous oil. The biological assay of the alcohol obtained from various allophanate preparations is given in Table I.

Isolation of 138° Allophanate—The methyl alcoholic mother liquor from the first crude separation of the allophanates was allowed to stand 4 weeks at 0°. During this time the crystalline allophanate slowly separated, and was then filtered off and recrystallized several times from ethyl and methyl alcohol. By

dissolving it in 50 volumes of hot 95 per cent ethyl alcohol and allowing the solution to cool slowly, about four-fifths of the allophanate separated as beautiful needles several mm. long. Although this allophanate separates very much more slowly than that of α-tocopherol, it is, if anything, less soluble in ethyl alcohol and has little or no tendency to be accompanied by oil, so that its purification is much less bothersome.

Analysis—Heating at 80° *in vacuo* caused no loss of weight.

	I	II	II
C	72.05	72.01	72.00
H	10.02	10.15	10.19
N		5.39	5.39

Calculated for $C_{31}H_{52}N_2O_4$. C 72.04, H 10.15, N 5.42

The analysis under I was made by Dr. Schoeller; under II at Columbia University.

Fractional Adsorption of α-Tocopheryl Allophanate—100 gm. of Baker's Analyzed $CaCO_3$ powder, which had been heated for 3 hours at 150°, was packed tightly into a 35 mm. tube, as described by Strain (15), except that supercel was not necessary.   106 mg. of a sample of α-tocopheryl allophanate, m.p. 156–157°, dissolved in a small volume of benzene were introduced, and the chromatogram developed with benzene.   The first 400 cc. of filtrate were evaporated to dryness under reduced pressure, and left no residue. The following 750 cc. washed through 26 mg. of substance, which on recrystallization from a small amount of alcohol weighed 20.2 mg., m.p. 157–158°.   This was hydrolyzed in the usual way, yielding Preparation 110-D; the results with feeding this are shown in Table I.

The filtration was then discontinued, and the column pushed out of the tube, divided into three approximately equal sections, and eluted with a mixture of alcohol and benzene.   The yield and melting point of the eluates are as follows:

Portion of column	Weight of crude material	Weight of recrystallised material	M.p.
	mg.	*mg.*	*°C.*
Bottom	63	49.2	158–159
Middle	18 *Ca.*	12.0	157–158
Top	1		

The allophanate from the middle section of the column was hydrolyzed in the usual way, yielding 9 mg. of α-tocopherol (Table I, Preparation 112-A).

Conversion of α-Tocopheryl Allophanate to the p-Nitrophenylurethane—500 mg. of the allophanate were hydrolyzed in the usual way, and the alcohol heated on the steam bath for an hour with 1 gm. of *p*-nitrophenyl isocyanate, no solvent being used. The reaction mixture was dissolved in benzene, and the excess reagent destroyed by adding acetone containing a small amount of water. After standing 24 hours, the di-*p*-nitrophenylurea was filtered off, and the residue taken up in petroleum ether. Some *p*-nitraniline separated. The filtrate was evaporated to dryness and taken up in methanol, from which, on cooling, the nitrophenylurethane separated. After several recrystallizations it melted at 129–131°, though not very sharply.

Dr. Adolf Pabst of the Geology Department very kindly examined the crystals of α-tocopheryl *p*-nitrophenylurethane and described them thus:

"Very fine needle-like crystals. Parallel extinction, moderate to strong birefringence, biaxial positive, $2V$ small to moderate. Always shows interference figures normal to axial plane between optic axis and the obtuse bisectrix, with plane of the optic axes normal to the elongation of crystals. Elongation always negative. $\beta = 1.514 \pm 0.002$. The other indices could not be determined with certainty, but it is probable that α is near 1.51 and γ near 1.53. It is highly probable that the crystals are orthorhombic, though they may be monoclinic or triclinic."

Analysis of α-Tocopheryl-p-Nitrophenylurethane—The analysis was made by Dr. Ing. A. Schoeller, Berlin. The substance, dried at 80° *in vacuo*, showed no loss of weight.

C	73.02	73.09	72.98
H	9.34	9.41	9.21
N	5.01	5.05	5.02

Calculated for $C_{36}H_{54}N_2O_5$. C 72.67, H 9.15, N 4.71
"   " $C_{37}H_{56}N_2O_5$. " 72.98, " 9.27, " 4.60

Hydrolysis of the Nitrophenylurethane—The nitrophenylurethane was hydrolyzed with 4 per cent methyl alcoholic potassium hydroxide, as described for the allophanate. The ether residue was taken up in petroleum ether, from which a considerable amount of nitraniline separated and was filtered off. The remainder was

removed by shaking a few times with 50 per cent methyl alcohol (Table I, Preparation 90-B).

Reconversion of Alcohol from p-Nitrophenylurethane to Allophanate—200 mg. of the *p*-nitrophenylurethane were hydrolyzed as described, and the alcohol, freed from *p*-nitraniline, was dissolved in benzene and saturated with cyanic acid gas from 2 gm. of cyanuric acid. After standing 4 days, the allophanate was worked up in the usual manner, and after a few recrystallizations from ethyl alcohol, melted at 158–160°.

50 mg. of the allophanate were hydrolyzed in the usual manner and used for biological assay and spectroscopic measurement (Table I, Preparation 146-A; Fig. 1, Curve 1).

SUMMARY

• We have prepared from the non-saponifiable matter of wheat germ oil three allophanates:

1. M.p. 250°. This is possibly the allophanate of β-amyrin. The alcohol regenerated from the allophanate has no vitamin E potency:

2. M.p. 138°, readily crystallizing in long needles. The analysis agrees with values required by monoallophanates of an alcohol, $C_{29}H_{50}O_2$. The alcohol from this allophanate apparently has some vitamin E potency, but less than that from the third allophanate.

3. M.p. 158–160°. From this allophanate, the alcohol—for which we propose the name *α-tocopherol*—when given in a single dose of 3 mg. always enables vitamin E-deficient rats to bear young. α-Tocopherol shows a characteristic absorption band at 2980 Å., $E_{1\,cm.}^{1\,per\,cent} = 90\ ca.$ Treatment with methyl alcoholic silver nitrate converts it to a substance which has absorption bands at 2710 and 2620 Å. respectively, $E_{1\,cm.}^{1\,per\,cent} = 480\ ca.$, and possesses some vitamin E activity.

α-Tocopherol yields a crystalline *p*-nitrophenylurethane melting at 129–131°. Analyses of both the urethane and the allophanate indicate a provisional formula for α-tocopherol of $C_{29}H_{50}O_2$.

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A NEW AND EASY METHOD FOR THE POTENTIOMETRIC DETERMINATION OF CALCIUM CONCENTRATIONS IN SOLUTIONS

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It has been shown in a previous paper (1) that minerals can •be used as materials for electrodes. I shall now describe more in detail researches on fluorite as a calcium electrode.

Fluorite, CaF_2, is a mineral which can be obtained in beautiful crystals. From a crystal a thin plate is split off, which is sealed on a glass tube by means of sealing-wax or some other substance not reacting with water. After being sealed the plate is ground and polished to make it as thin as possible, care being taken that the plate does not become porous. The tube is then placed for 24 hours with the open end in a beaker of water; if the water rises in the tube, the electrode is porous.

Inside the tube is placed a solution of calcium chloride, *e.g.* 0.05 M; in this solution a wire of silver, covered with silver chloride, is placed. The silver-silver chloride electrode was prepared as follows:

A platinum wire is covered by a silver layer by electrolysis (4 volts, 1 milliampere) of a solution obtained by mixing equal volumes of 13 per cent KCN solution and 18 per cent $AgNO_3$ solution. After washing, the silver-plated platinum wire is electrolytically covered with a thin layer of AgCl, a 1 N solution of HCl and a current of 3.5 milliamperes being used for 20 minutes. Then the electrode is thoroughly washed with hot distilled water.

The calcium electrode is then checked in solutions of $Ca(NO_3)_2$. The standard solution of calcium nitrate was analyzed by oxidimetric titration with $KMnO_4$ of the calcium oxalate precipitated in the ordinary way.

From the standard solution dilutions were made and the E.M.F. of the cell

Ag, AgCl | CaCl₂ | CaF₂ | Ca(NO₃)₂ | KCl (saturated) | KCl (0.1 M),
                                Hg₂Cl₂ | Hg
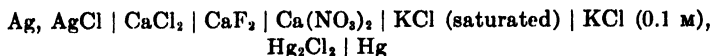
was measured at room temperature.

The E.M.F. was measured with a Leeds and Northrup Students' potentiometer. Since the resistance is very great, it is necessary to use vacuum tubes. We used two tubes and a scheme given by Janssen (2). The first tube was a Philips No. 4060; the anode current of the second tube (Philips No. B-405) was counteracted by a current so as to make the galvanometer a null point indicator.

When measurements are to be made, it is necessary first of all

TABLE I

Relation between Electromotive Force and Calcium Concentration

Concentration of Ca nitrate	$pCa = - \log c$	E.M.F. of cell
m -eq. per l.		*mv.*
1000	0	68.5
100	1	54.5
10	2	41.0
1	3	28.5
0.1	4	13.5
0.01	5	12.0
0.001	6	12.0

to check the CaF₂ electrode with known calcium nitrate solutions. The equilibrium is attained in a few minutes. By way of example I give the results with Electrode 35 in Table I.

Fig. 1 shows the curve of the E.M.F. of the cells plotted against the values of pCa. There is a linear relation between pCa and the E.M.F. but deviations occur at pCa 5 and higher. This is probably due to the solubility of CaF₂ itself.

The solubility product is

$$[Ca^{++}] [F^-]^2 = 3.4 \times 10^{-11} \text{ (at } 18°)$$
$$[Ca^{++}] = 3.2 \times 10^{-4}$$

Thus in a saturated solution of CaF₂ the calcium concentration is 0.64 milli-equivalent per liter. This means that the CaF₂ elec-

trode itself, placed in solutions of calcium nitrate weaker than 0.64 milli-equivalent per liter, gives rise to a higher concentration which remains practically constant, thus making all measurements misleading. Therefore, it is impossible to use concentrations weaker than about 1 milli-equivalent per liter.

As it is of great importance to know the distribution of calcium in protein sols, we tried the method in the study of gelatin sols and milk.[1] The adsorption of calcium by gelatin has been studied by Eversole, Ford, and Thomas (3).

Isoelectric gelatin, prepared from the purest gelatin of the Lym-en Gelatinefabriek, Delft, was dissolved and by adding hydrochloric acid or sodium hydroxide was brought to different pH
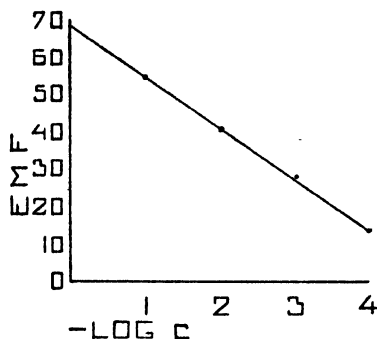
Fig. 1. Relationship between E.M.F. and calcium concentration

values. The pH was measured with a glass electrode; the glass was from the Corning Glass Works, made according to the specifications of MacInnes.

The measurements were made with 1 per cent gelatin sols of pH 4.32, 4.55, and 5.40. First of all, it was found that the CaF_2 electrode showed exactly the same potential in each of these three gelatin sols. This means that the potential of this electrode is not influenced by hydrogen ions.

The mixtures of gelatin and calcium nitrate were made by adding 25 cc. of calcium nitrate solution to 25 cc. of gelatin sol. In a few minutes a constant E.M.F. is obtained; after each measurement the electrode is thoroughly rinsed with distilled water.

[1] These experiments on gelatin and milk were carried out in collaboration with Mr. B. M. Krol.

The measured E.M.F. of the cell gives the pCa $= -\log c$, which can be read from the curve of Fig. 1. It is necessary to standardize every time before a series of measurements; sometimes small

TABLE II

Calcium-Binding Power of Gelatin

Ca-(NO₃)₂ added	Gelatin, pH 5.40			Gelatin, pH 4.55			Gelatin, pH 4.32		
	pCa	Ca concentration	Ca bound by 10 gm. gelatin	pCa	Ca concentration	Ca bound by 10 gm. gelatin	pCa	Ca concentration	Ca bound by 10 gm. gelatin
m.-eq. per l.		*m.-eq. per l.*	*m.-eq*		*m.-eq. per l.*	*m.-eq.*		*m.-eq. per l.*	*m.-eq.*
364	1.10	89.1	274 9	0.85	158.5	205.5	0.60	251.2	112 8
	0 97			0.70			0.57		
182	1.30	56.2	125 8	0.95	125 9	56 1	0 85	141 2	40 8
	1.20			0 83			0.80		
91	1.50	31.6	59 4	1.10	79.4	11.6	1.15	79 4	11.6
	1.45			1 05			1 05		
36 4	1.87	12 6	23.8	1 47	31.6	4.8	1.57	31.6	4 8
	1.93			1.55			1.45		
18.2	2.33	5.0	13 2	1 93	12 6	5.6	1.87	14 1	4 1
	2.25			1 87			1.85		
9 1	2.50	3.2	5.9	2 25	5 6	3.5	2.13	7 7	2 0
	2.45			2 20			2.17		
7.3	2.80	1.6	5 7	2.45	3 6	3.7	2.33	4 5	2.8
	2.80			2.40			2.40		

TABLE III

Influence of Gelatin Concentration on Calcium Adsorption

Concentration of gelatin	pCa	Ca concentration	Ca bound	Ca bound per gm. gelatin
per cent		*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq.*
2½	1.35	44.7	455.3	18.2
2	1.20	63.1	436.9	21.8
1½	0.90	125.9	374.1	24.9
1	0.80	158.5	341.5	34.1
½	0.50	316.2	183 8	37.1

differences may occur, depending on the room temperature. All determinations have been made twice. From the figures for pCa one sees that the reproducibility is very good. The results are presented in Table II.

From these results it follows that calcium is bound or adsorbed by gelatin. As it is well known that at pH >4.7 the gelatin is negative, the binding of calcium will also be greater than at pH <4.7, where gelatin is positive. From the figures it is clear that the binding of calcium becomes less on lowering the pH. The amount of calcium which is adsorbed by gelatin is fairly great.

Also the influence of the concentration of gelatin, the concentration of calcium nitrate being kept constant at 500 milli-equivalents per liter, has been studied. The results are given in Table III.

From these results it follows that the adsorption of calcium per gm. of gelatin is greater the smaller the gelatin concentration. This fact could be expected *a priori*.

Attention was paid next to the adsorption of calcium in milk. The amount of soluble calcium varies, according to Van Slyke and Bosworth (4) between 0.0343 and 0.0734 per cent. The amount of soluble calcium is that part of the total calcium content present which can be measured with the CaF_2 electrode. This amount must be practically equal to the amount of calcium in the milk serum. First of all I determined the pH and pCa of a sample of fresh milk; afterwards the milk was filtered through a collodion membrane, and the pH and pCa of the ultrafiltrate were again determined. The following results were obtained.

	pH	pCa	Calcium	
			m.-eq. per l.	*per cent*
Whole milk....................	6.63	1.47	33.9	0.068
Milk serum...................	6.73	1.45	35.5	0.071

Though the calcium concentrations are not exactly the same, it is justifiable to regard them as equal. This control proves that the method is reliable.

The pH of milk serum is higher than the pH of whole milk. This must be ascribed to the well known "suspension effect" of Wiegner. It might be possible that on further study of the calcium concentrations under different conditions a suspension effect for this ion will be found.

Next, different amounts of calcium nitrate were added to milk. The mixtures were made by mixing 10 cc. of milk and 10 cc. of a

calcium nitrate solution; also a mixture of 10 cc. of milk and 10 cc. of water was made. Similar measurements were made with the same sample of milk which had been previously acidified with

TABLE IV

Calcium-Binding Power of Milk

A is the concentration of calcium in milk diluted with 1 volume of water, as measured with the CaF_2 electrode; B, the amount of calcium, added as nitrate; C, the concentration of calcium in the mixtures with calcium nitrate, as measured with the electrode (A, B, and C are expressed in milli-equivalents per liter). Thus $C - A$ is the increase of the calcium concentration in the intermicellar liquid, and $B - (C - A)$ is the amount of calcium adsorbed.

Ca(NO$_3$)$_2$ added (B)	Fresh milk					Acidified milk				
	pH	pCa	Ca concentration found (A)	$C - A$	$B - (C-A)$	pH	pCa	Ca concentration found (A)	$C - A$	$B - (C-A)$
m.-eq. per l.			*m.-eq. per l.*					*m.-eq. per l.*		
0	7.14	1.80	15 8			5.98	1 63	25.1		
	7.20	1.80				5.93	1.60			
			(C)					*(C)*		
364	5 78	0.83	147 9	132 1	231.9	4.80	0.60	251.2	226.1	137.9
	5 78	0.83				4.78	0.58			
182	5.93	1 10	79.4	63 6	118.4	5.00	0.83	141.2	116 1	65.9
	6 00	1.08				5.00	0.88			
91	6.23	1 28	52.5	36.7	54.3	5.15	1.08	89.1	64.0	27.0
	6.15	1 25				5.18	1 00			
36.4	6.55	1.65	25.1	9.3	26.7	5.45	1.40	39.8	14.7	21.7
	6.63	1.58				5.38	1.35			
18 2	6.85	1 80	15 8	0	18.2	5.55	1.60	25.1	0	18.2
	6.85	1.78				5.63	1.58			
9.1	6.98	1.80	15.8	0	9.1	5.85	1.60	25.1	0	9.1
	7.05	1.80				5.70	1.60			
7.3	6.98	1.80	15 8	0	7.3	5.80	1.60	25.1	0	7.3
	7.05	1.80				5.80	1.60			

lactic acid. All mixtures were made twice. In Table IV the results are presented. From these data it follows that the reproducibility of the measurements is very good. The mean values of pCa are used in the calculations.

The adsorption of calcium is less in the acidified milk. Small amounts of calcium added are completely adsorbed. From Table I it can be seen that between 100 and 10 milli-equivalents every 10 milli-equivalents correspond with 1.35 millivolts, and between 10 and 1 milli-equivalents every milli-equivalent corresponds with 1.25 millivolts. Therefore, a difference in adsorption by adding 18.2 or 7.3 milli-equivalents of calcium nitrate per liter respectively would have been detectable if the adsorption had not been complete. The fact that no difference occurs proves that the added calcium is completely adsorbed.

It is open to discussion whether the calcium is adsorbed, or bound, by casein or the phosphates. In every case we may conclude that milk behaves as a calcium buffer.

SUMMARY

A method has been developed to determine potentiometrically concentrations of calcium in solutions and in protein sols. The CaF_2 electrode can be used at calcium concentrations of 1 milli-equivalent per liter and higher.

It has been shown that calcium is adsorbed by proteins and that the adsorption decreases with increasing acidity of the protein sols.

The use of CaF_2 as an electrode provides the great advantage that no foreign substances are introduced into the systems to be studied, as is the case when electrodes of the second or third order are used.

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A METHOD FOR THE STEPWISE DEGRADATION OF POLYPEPTIDES

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In the analysis of proteins one of the problems encountered is .the determination of the arrangement of the amino acid residues in polypeptides. A solution to this problem has been sought by protein chemists for a long time. Fischer and Abderhalden (1) hydrolyzed dipeptides in which the free amino group was substituted by means of a naphthalenesulfonyl residue; the amino acid bearing the free amino group in the dipeptide was isolated from the hydrolysate as the naphthalenesulfonyl derivative. Several authors (2, 3) later used analogous methods in order to label a terminal amino acid in a peptide and to isolate its derivative following hydrolysis. The phenyl isocyanate method described by Bergmann, Miekeley, and Kann (3) was further developed and applied by Abderhalden and Brockman (4) in order to determine the order of amino acids in the tripeptide *dl*-alanylglycyl-*dl*-leucine.

In what follows, a method is described whereby each successive amino acid in polypeptides may be characterized and the order of amino acids in the polypeptide chain determined. The method involves a combination of the azide degradation of Curtius with our carbobenzoxy method (5). The general principle of the method was recently described (6), the phenyl isocyanate

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† The preliminary experiments for this research were carried out 2 years ago in the Kaiser Wilhelm Institute for Leather Research, Dresden, Germany, where we enjoyed the collaboration of Dr. Ferdinand Schneider. His experiments are described in his doctoral dissertation, Munich, 1935.

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compound of the tripeptide glycyl-*l*-alanyl-*l*-leucine being used. Fischer and Waibel (7) found the same carbobenzoxy degradation in experiments with pyrrolecarboxylic acids.

It was found during the further development of the peptide degradation that the best results were obtained by employing benzoylated peptides. The method will therefore be described for the benzoyl derivative of the tetrapeptide glycyl-*l*-alanyl-*l*-leucyl-*l*-glutamic acid. As an introduction, there is described the carbobenzoxy degradation of the benzoyl derivatives of *l*-leucine, *dl*-phenylalanine, and *l*-glutamic acid.

When an amino acid (I) is to be degraded according to the carbobenzoxy method, it is first benzoylated at the α-amino group (II) and converted into the azide (III) through the methyl ester and hydrazide. On slight heating with benzyl alcohol, this azide forms the carbobenzoxy compound, benzylurethane (IV), which may be catalytically hydrogenated in a hydrochloric acid solution to the hydrochloride of the amine (V). This type of compound belongs to the derivatives of doubly aminated aldehydes. They are sufficiently stable to be isolated in a well crystallized form and therefore deserve closer investigation.

For the purpose of this work it was important that on warming with water these derivatives of doubly aminated aldehydes are split to benzamide, ammonium chloride, and the aldehyde (VI) and can easily be characterized in the form of derivatives.

$$\begin{aligned}
&\text{I. } R \cdot CH(NH_2) \cdot COOH \\
&\text{II. } R \cdot CH(NH \cdot Bz) \cdot COOH \\
&\text{III. } R \cdot CH(NH \cdot Bz) \cdot CON_3 \\
&\text{IV. } R \cdot CH(NH \cdot Bz) \cdot NH \cdot CO \cdot O \cdot CH_2 \cdot C_6H_5 \\
&\text{V. } R \cdot CH(NH \cdot Bz) \cdot NH_2 \\
&\text{VI. } R \cdot CHO \\
&\qquad\qquad Bz = C_6H_5 \cdot CO
\end{aligned}$$
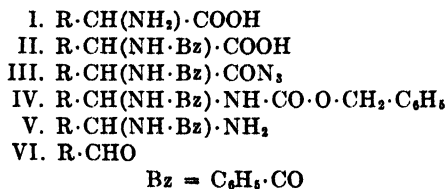
By this method isovaleraldehyde is obtained from *l*-leucine, while phenylalanine yields phenylacetaldehyde.

As is to be expected, aminodicarboxylic acids are degraded in two positions. Benzoylglutamic acid (VII) gives, for example, the diazide (VIII) and then the dicarbobenzoxy compound (IX). The last named compound is hydrogenated to (X), which on splitting yields β-aminopropionaldehyde (XI). It was found that

this aldehyde may well be characterized as the dimethone derivative.

VII. $HO \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot COOH$
VIII. $N_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot CO \cdot N_3$
IX. $C_6H_5 \cdot CH_2 \cdot O \cdot CO \cdot NH \cdot CH_2 \cdot CH_2(NH \cdot Bz) \cdot NH \cdot CO \cdot O \cdot CH_2 \cdot C_6H_5$
X. $NH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot NH_2$
XI. $NH_2 \cdot CH_2 \cdot CH_2 \cdot CHO$

Degradation experiments with diaminocarboxylic acids have not yet been performed. These should, however, also give amino aldehydes.

It should be pointed out that in the case of optically active amino acids all the reaction products up to (V) inclusive, and similarly (VII to X), retain the optical activity. This corresponds to the finding of Wallis (8) that optically active carboxylic acids give optically active amines on degradation.

Curtius (9) had already attempted the degradation of benzoylated amino acids but was forced to split the urethanes, obtained with methyl or ethyl alcohol (analogous to (IV)), by energetic treatment with acids. The resulting aldehydes and acid amides were usually decomposed under these conditions. Therefore, the original Curtius method could not be used in peptide chemistry. The carbobenzoxy degradation will also be useful for the degradation of other carboxylic acids, when it is necessary to isolate labile degradation products under mild conditions.

In order to perform the carbobenzoxy degradation on a tetrapeptide, glycyl-*l*-alanyl-*l*-leucyl-*l*-glutamic acid was synthesized by the carbobenzoxy method and then the individual amino acids were successively split off and identified in the form of the corresponding aldehydes with 1 carbon atom less. In this degradation the intermediate steps shown in the accompanying diagram were involved.

The conversion of the intermediate amide into the hydrazide was performed by the direct action of hydrazine.[1]

In its present form the carbobenzoxy degradation should be applicable to the identification of every α-amino acid in peptides.

[1] This reaction usually involves losses. It will therefore be attempted in the future to transform the amides into the carboxylic acids with nitrous acid, and to secure the hydrazide through the ester.

Benzoylglycylalanylleucylglutamic acid

> Di-ester
> Dihydrazide
> Diazide
> Dicarbobenzoxy compound
>
> | Hydrogenation and splitting
> ↓

Benzoylglycylalanylleucineamide + β-aminopropionaldehyde

> Hydrazide
> Azide
> Carbobenzoxy compound
>
> | Hydrogenation and splitting
> ↓

Benzoylglycylalanineamide + isovaleraldehyde

> Hydrazide
> Azide
> Carbobenzoxy compound
>
> | Hydrogenation and splitting
> ↓

Benzoylglycineamide + acetaldehyde

Further special experiments are necessary on the behavior of proline and hydroxyproline.

We wish at this point to acknowledge the kind assistance of Dr. Joseph S. Fruton in preparing this manuscript, and of Mr. J. Goldberg and Dr. A. Elek in performing the analyses.

Degradation of l-Leucine

N-Benzoyl-l-Leucine Methyl Ester—10 gm. of *l*-leucine were suspended in 50 cc. of dry methanol which was then saturated with HCl without cooling. After evaporation, the esterification was repeated. From the hydrochloride an ethereal solution of the free ester was prepared in the usual manner, and to the latter there were added, at 0°, 6 cc. of benzoyl chloride in 50 cc. of ethyl acetate. Then 6 cc. more of benzoyl chloride and 100 cc. of 10 per cent sodium carbonate solution were added at 0° in several portions, with shaking. The ether-ethyl acetate solution was

washed in turn with pyridine, water, hydrochloric acid, and potassium bicarbonate, then dried and evaporated down, yielding crystals which were transferred to the filter with cold ether. A second crystallization was obtained by precipitation with petroleum ether. Yield, 10.6 gm. After recrystallization from ether the melting point was 104°.

$C_{14}H_{19}O_2N$ (249.2). Calculated, N 5.6; found, 5.5[a]

Benzoyl-l-Leucine Hydrazide—8.5 gm. of the above ester were dissolved in 15 cc. of hot alcohol and 2.5 cc. of hydrazine hydrate, and allowed to stand 24 hours at room temperature. The needles which separated out were washed with ice-cold water. Yield, 7.5 gm. On recrystallization from alcohol, the melting point was 153°.

$C_{13}H_{19}O_2N_3$ (249.2). Calculated, N 16.9; found, N 17.0

1-Benzamido-1-Carbobenzoxyamido-3-Methylbutane—7 gm. of the above hydrazide were dissolved in a mixture of 10 cc. of 5 N hydrochloric acid and 40 cc. of 50 per cent acetic acid; the solution was diluted with 150 cc. of water, cooled to 0°, and an aqueous solution of 2.2 gm. of sodium nitrite was added within 2 minutes. The sirupy precipitate of azide was extracted with a quantity of ether, the ether solution was washed four times with ice-cold water, then with bicarbonate, and again with water, filtered, dried over sodium sulfate, and 10 cc. of benzyl alcohol were added. The ether was evaporated *in vacuo* and the resulting solution heated to 70–80° (nitrogen generated). The crystals which separated out were washed with ether and filtered. Yield, 3 gm. The substance was recrystallized from glacial acetic acid. Needles; m.p., 178°.

$C_{20}H_{24}O_3N_2$. Calculated. C 70.6, H 7.1, N 8.2
340.2   Found.   " 70.6, " 7.2, " 8.0

1-Benzoylamino-1-Amino-3-Methylbutane—1 gm. of the carbobenzoxy compound was suspended in methanol which contained 1.1 moles of 5 N aqueous hydrochloric acid, and hydrogenated with palladium catalyst in an open vessel. The hydrogenation

[a] The nitrogen determinations reported in this paper were carried out by the micro-Dumas method except in the two instances in which the micro-Kjeldahl method is designated.

and carbon dioxide regeneration were complete after about 20 minutes. On evaporation under diminished pressure (bath temperature 35°), flat needles of the hydrochloride were obtained, which were transferred to the filter with acetone-ether. Yield, 0.6 gm.

$[\alpha]_D^{18}$ = −47° (5 per cent in methanol)

$C_{12}H_{19}ON_2Cl$.   Calculated.   C 59.4, H 7.9, N 11.5, Cl 14.6
242.6   Found.   " 59.7, " 7.9, " 11.4, " 14.7

The base can be liberated from the hydrochloride with sodium hydroxide and can be taken up in ether. On passing hydrochloric acid gas into the ethereal solution, the hydrochloride separates out immediately. On boiling the hydrochloride with methanol there crystallized out after a short time needles (in good yield) which contained no chlorine and which had a melting point of 210°. On recrystallization from methanol no elevation of the melting point was observed. The analysis indicates that the substance is the dibenzamido derivative of isovaleraldehyde.

$(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5)_2$

$C_{19}H_{22}O_2N_2$.   Calculated.   C 73.5, H 7.2, N 9.0
310.2   Found.   " 73.3, " 7.2, " 9.0

Splitting of the Hydrochloride—0.3 gm. of the above hydrochloride was dissolved in 5 cc. of water and the solution distilled in 5 minutes from a bath of 120° into an ice-cooled receiving flask which contained 0.3 gm. of *p*-nitrophenylhydrazine in 5 cc. of 50 per cent acetic acid. In the receiving flask there separated out the *p*-nitrophenylhydrazone of isovaleraldehyde (after recrystallization 0.26 gm. of needles of melting point 113° was obtained (10); calculated, N 19.0; found, N 19.2). In the distilling flask benzamide (0.1 gm., m.p., 127°; mixed m.p. with commercial benzamide, 127°) separated out, while the ammonium chloride remained in solution.

Degradation of dl-Phenylalanine

Benzoyl-dl-Phenylalanine Methyl Ester—From 10 gm. of phenylalanine there were obtained 14 gm. of benzoyl ester by the same method employed for leucine. On recrystallization from ether the melting point was 90°.

$C_{17}H_{17}O_3N$ (283.1). Calculated, N 4.9; found, N 5.0

Benzoyl-dl-Phenylalanine Hydrazide—A solution of 14 gm. of the above ester in 30 cc. of warm alcohol was treated overnight with 4 cc. of hydrazine hydrate at room temperature. On working up the solution, 12.5 gm. of recrystallized hydrazide (m.p., 192°) were obtained.

$C_{16}H_{17}O_2N_3$ (283.1). Calculated, N 14.8; found, N 15.1

1-Carbobenzoxyamino-1-Benzamino-2-Phenylethane—8 gm. of the above hydrazide were dissolved in 50 cc. of 50 per cent acetic acid and 5 cc. of 5 N hydrochloric acid, the solution was diluted with 150 cc. of water and cooled to 0°, and an aqueous solution of 2 gm. of sodium nitrite added within 2 minutes. After about 3 minutes, the precipitated azide was taken up in ethyl acetate, washed three or four times with ice-cold water, quickly with bicarbonate, and again with water, filtered, and dried over sodium sulfate. 20 cc. of benzyl alcohol were then added and the ethyl acetate was removed under reduced pressure at 50°; then the solution was heated on the steam bath for ½ hour. The crystals which separated out were treated with glacial acetic acid and filtered with suction. Yield, 5.9 gm. On recrystallization from glacial acetic acid, rods with a melting point of 196° were obtained.

$C_{23}H_{22}O_3N_2$. Calculated. C 73.7, H 5.9, N 7.5
374.2   Found.   " 73.6, " 6.1, " 7.4

Hydrogenation to 1-Amino-1-Benzamido-2-Phenylethane—5.5 gm. of carbobenzoxy compound were suspended in 100 cc. of methanol and 5 cc. of 5 N HCl, and hydrogenated in the usual manner with palladium (about 2 gm.) as catalyst. After hydrogenation, the filtrate was evaporated down at 35° and the residue transferred to the filter with acetone-ether. A spongy mass of needles was obtained; yield, 3 gm.

$C_{15}H_{17}ON_2Cl$. Calculated. C 65.1, H 6.2, N 10.1, Cl 12.8
276.6   Found.   " 64.8, " 6.0, " 10.2, " 13.0

If the hydrochloride is dissolved in water and sodium hydroxide or ammonium hydroxide is added, the free base separates out in nearly quantitative yield. Needles; m.p., 156° (after browning and sintering).

$C_{15}H_{16}ON_2$. Calculated. C 75.0, H 6.7, N 11.7
240.1   Found.   " 75.0, " 6.7, " 11.5

Deamination of the Amine—0.5 gm. of the hydrochloride was dissolved in water containing several drops of acetic acid. On addition of an aqueous solution of 0.18 gm. of sodium nitrite, there began nitrogen generation which ended after a short time. The precipitated needles (0.35 gm.) were recrystallized from methanol-water. M.p., 128°.

<div align="center">

$C_{15}H_{15}O_2N$.   Calculated.   C 74.7, H 6.3, N 5.8
241.1   Found.   " 74.9, " 6.3, " 5.9

</div>

The substance is not affected by boiling water.

Splitting of the Amine—0.8 gm. of the above hydrochloride was dissolved in 10 cc. of water and after the addition of 0.6 gm. of sodium acetate and 0.2 gm. of hydroxylamine hydrochloride, heated to 100° for 15 minutes. On cooling, 0.3 gm. of phenylacetaldehyde oxime was obtained. Needles with a melting point of 103° (11) resulted after recrystallization from ether-petroleum ether.

<div align="center">

C_8H_9ON (135.1).   Calculated, N 10.4; found, N 10.4

</div>

On concentrating the mother liquor *in vacuo*, 0.2 gm. of benzamide was obtained as plates (m.p., 127°). The mixed melting point with commercial benzamide showed no depression.

<div align="center">

Degradation of l-Glutamic Acid

</div>

Benzoyl-l-Glutamic Acid Dimethyl Ester—This was prepared from 14.7 gm. of glutamic acid in the same way as for leucine (instead of soda, potassium bicarbonate was employed). Yield, 25 gm. On recrystallization from ether, needles, m.p. 83°, were obtained.

<div align="center">

$C_{14}H_{17}O_5N$ (279.1).   Calculated, N 5.0; found, N 4.8 (micro-Kjeldahl)

</div>

Benzoyl-l-Glutamic Acid Dihydrazide—From the solution of 25 gm. of the dimethyl ester in 75 cc. of absolute alcohol to which 11 cc. of hydrazine hydrate were added, there separated out on standing overnight 20 gm. of dihydrazide. The substance was recrystallized from alcohol. Needles; m.p., 216°.

<div align="center">

$C_{12}H_{17}O_3N_5$ (279.2).   Calculated, N 25.1; found, N 24.9

</div>

Benzoyl-l-Glutamic Acid Diazide—8 gm. of dihydrazide were dissolved in 130 cc. of hot water, 4.3 gm. of sodium nitrite were added, and the solution was cooled to 0°; this was followed by the addition of 31 cc. of 2 N HCl within 1 minute. After about 3 to 5 minutes at 0°, the supernatant solution was discarded and the semisolid precipitate treated with about 2 cc. of ether, whereupon it crystallized. It was transferred to the filter with a little cold water, dried on a porous plate, and then over sulfuric acid *in vacuo.* Yield, 5.6 gm. of crystals which effervesced around 75°.

1,3-Biscarbobenzoxyamino-1-Benzaminopropane—4.8 gm. of diazide were added to 20 cc. of xylene and 12 cc. of benzyl alcohol, and heated slowly in a paraffin bath. Nitrogen generation began around 60–70°. The reaction mixture was kept at 70–80° until the nitrogen formation had slowed down, whereupon the temperature was raised to the boiling point of xylene for 15 minutes. On adding ether, 4.2 gm. of a spongy crystalline substance were obtained. It was dissolved in 30 cc. of hot 50 per cent acetic acid and allowed to cool slowly. The yield is decreased by one-half. Hair-like needles were obtained; m.p., 174°.

$C_{26}H_{27}O_5N_3$.   Calculated.   C 67.6, H 5.9, N 9.1
461.2   Found.   " 67.5, " 6.1, " 9.2

Hydrogenation of the Dicarbobenzoxy Compound—2 gm. of the dibenzylurethane were suspended in absolute methanol which contained 2.1 moles of hydrogen chloride. The hydrogenation in the presence of 0.5 gm. of palladium catalyst was completed in 15 minutes. On evaporation *in vacuo* at 30°, the crystalline hydrochloride remained behind. Yield, 0.8 gm. Recrystallization from cold methanol-ether gave rods which melted at 158° with sintering. $[\alpha]_D^{22} = -50.7°$ (6.9 per cent in water). Owing to the instability of the dihydrochloride, the analysis was performed on the dipicrate of the diamino compound.

The dipicrate can be obtained in quantitative yield from an aqueous solution of the hydrochloride by adding sodium picrate. It crystallizes from hot water containing picric acid in long rods which decompose slowly at 100°.

$C_{21}H_{31}O_{15}N_9 \cdot H_2O$.   Calculated.   C 39.4, H 3.5, N 18.8
669.2   Found.   " 39.4, " 3.4, " 18.7

The instability of the substance on heating prevented a determination of the water of crystallization.

Splitting of the Diamine—0.4 gm. of dimethone was dissolved in the required volume of water, and 0.4 gm. of the dihydrochloride added to the solution, which was then heated to boiling for 5 minutes. On cooling, the solution was extracted with a 1:1 mixture of ethyl acetate-ether, followed by ether extraction. The extracts were washed with a small volume of dilute sodium carbonate solution (to remove free dimethone) and concentrated down. 0.22 gm. of the benzamide in the form of square plates (m.p., 127° after recrystallization from water) was obtained. This showed no melting point depression with commercial benzamide. The aqueous solution (see above) contained the dimethone compound of β-aminopropionaldehyde as the hydrochloride. This substance was precipitated by making the solution slightly alkaline with sodium hydroxide and acidifying again with 1 or 2 drops of acetic acid. On standing overnight, 0.25 gm. of the dimethone anhydride of β-aminopropionaldehyde was obtained, which after recrystallization from aqueous alcohol and then from absolute alcohol melted at 208–209°.

$C_{19}H_{27}O_3N$.   Calculated.   C 71.9, H 8.6, N 4.5
317.2   Found.   " 72.1, " 8.3, " 4.4

Synthesis of Glycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid

Carbobenzoxyglycyl-l-Alanine Ethyl Ester—To a solution of *l*-alanine ethyl ester in ethyl acetate (prepared from 15 gm. of the hydrochloride) there were added, in four portions with cooling, 11 gm. of carbobenzoxyglycyl chloride. With shaking, 11.5 gm. more of carbobenzoxyglycyl chloride were added in five portions, each of which was followed by 10 cc. of a 10 per cent sodium carbonate solution. After addition of about 100 cc. of water, the reaction mixture was shaken for a short time with 20 cc. of saturated bicarbonate solution. The aqueous layer was removed, the unchanged chloride decomposed with pyridine, and the ethyl acetate layer washed with water, dilute hydrochloric acid, and again with water, dried over sodium sulfate, and evaporated down. Upon scratching, the resulting sirup crystallized in long prisms. These were recrystallized from ether-petroleum ether. Yield,

22.5 gm.; m.p., 59° after two recrystallizations from ether-petroleum ether.

C₁₆H₂₀O₅N₂ (308.2).   Calculated, N 9.1; found, N 9.2

Carbobenzoxyglycyl-l-Alanine Hydrazide—22 gm. of the above ester were dissolved in 30 cc. of absolute alcohol and refluxed with 6 gm. of hydrazine hydrate for 1 hour.   On cooling, a crystalline mass was obtained which was washed with a little absolute alcohol and a quantity of ether.   Yield, 16.5 gm.; m.p., 133° after recrystallization from alcohol.

C₁₃H₁₈O₄N₄ (294.2).   Calculated, N 19.0; found, N 18.8

Carbobenzoxyglycyl-l-Alanine Azide—7.5 gm. of the above hydrazide were dissolved in 150 cc. of water and 17 cc. of 3.6 N HCl.   The solution was cooled to 0° and there were added solutions of 4.8 gm. of sodium acetate (containing water of crystallization) in 10 cc. of water and 1.9 gm. of sodium nitrite in 10 cc. of water, the latter being added dropwise.   The resulting sirup was taken up in ether, leaving a small portion undissolved.   The ethereal solution was washed often with ice-cold water, then with bicarbonate solution, and again with water, and dried over sodium sulfate.   This solution was used immediately for coupling.

Carbobenzoxyglycyl-l-Alanyl-l-Leucine Methyl Ester—To an ethereal solution of *l*-leucine methyl ester prepared from 14 gm. of the hydrochloride there was added the above ethereal solution of azide prepared from 20 gm. of the hydrazide.   After 12 hours, 8 gm. of the reaction product had separated out in needles.   The filtrate was concentrated to 100 cc., freed of crystals (0.5 gm.) which had separated out, washed with dilute hydrochloric acid, water, and bicarbonate, dried over sodium sulfate, and the substance precipitated with petroleum ether.   Total yield, 15 gm.; m.p., ·112° after recrystallization from ethyl acetate-petroleum ether.

C₁₉H₂₉O₆N₃ (407.2).   Calculated, N 10.3; found, N 10.4

Carbobenzoxyglycyl-l-Alanyl-l-Leucyl Hydrazide—5.7 gm. of the above tripeptide ester were dissolved in about 12 cc. of warm absolute alcohol, 0.8 cc. of hydrazine hydrate was added, and the solution allowed to stand overnight at room temperature.   The

needles which separated out were washed with a little alcohol.
Yield, 3.3 gm.; m.p., 186° after recrystallization from alcohol.

$C_{18}H_{26}O_6N_6$ (407.2). Calculated, N 17.2; found, N 17.5

*Carbobenzoxyglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dimethyl
Ester*—9 gm. of the hydrazide described above were dissolved in
225 cc. of water, 15 cc. of glacial acetic acid, and 22.5 cc. of N
HCl, and the solution was cooled to 0°. Then an aqueous solution
of 3 gm. of sodium acetate (with water of crystallization) was
added, followed by the dropwise addition (within 2 minutes) of
an aqueous solution of 1.65 gm. of sodium nitrite. The resulting
azide was taken up immediately in about 300 cc. of ethyl acetate,
the azide solution washed three times with ice-cold water, fol-
lowed by bicarbonate solution and again by water, filtered, and
dried quickly over a quantity of sodium sulfate. It was then
coupled with an ethereal solution of *l*-glutamic acid dimethyl
ester (prepared from about 9 gm. of glutamic acid). On concen-
trating the mixture and allowing it to stand overnight, needles
were obtained. On further concentration of the mother liquor, a
second crystallization resulted. Total yield, 7.2 gm.; m.p., 149°
after recrystallization from ethyl acetate.

$C_{24}H_{38}O_8N_4$ (550.3). Calculated, N 10.2; found, N 10.0 (micro-Kjeldahl)

Glycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid—2.7 gm. of the tetra-
peptide ester were suspended in 10 cc. of methanol, and with oc-
casional cooling and shaking saponified with 11 cc. of N sodium
hydroxide within 15 minutes. After 15 minutes more, the solu-
tion was made slightly acid to Congo red, the methanol removed
under reduced pressure, and the resulting sirup dissolved in a large
volume of ethyl acetate. The ethyl acetate layer was extracted
with bicarbonate. The bicarbonate extract was then acidified,
and the carbobenzoxytetrapeptide was again taken up in ethyl
acetate. After washing with water, the solution was evaporated
down, the residue dissolved in aqueous methanol and 0.3 cc. of
glacial acetic acid, and catalytically hydrogenated. The solution
was filtered, the catalyst washed frequently with water, and the
combined filtrate evaporated under reduced pressure. The crys-
tals (rhombic) were transferred to the filter with alcohol. Yield,

1.5 gm. On recrystallization from water the tetrapeptide forms long prisms which are transformed into rhombic crystals.

$C_{16}H_{29}O_7N_4 \cdot 1\frac{1}{2}H_2O$. Calculated. C 46.2, H 7.5, N 13.5, H_2O 6.5
415.2   Found.   " 45.9, " 7.6, " 13.3, " 6.3

3.77 mg. required 1.9 cc. of 0.01 KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 1.8 cc.

Degradation of the Tetrapeptide

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid—1 gm. of the free tetrapeptide was dissolved in a solution of 1.2 gm. of potassium bicarbonate in 10 cc. of water. Within 20 minutes there was added with shaking 0.35 cc. of benzoyl chloride in four portions. Each addition was preceded by cooling in ice. The shaking was performed at room temperature. On acidifying, the benzoyl compound separated out at first as a sirup; on scratching, however, it crystallized. After drying, the substance was boiled up with ether. Yield, 1.05 gm. Needles; m.p., 215° after recrystallization from aqueous alcohol.

$C_{23}H_{32}O_8N_4$. Calculated. C 56.1, H 6.5, N 11.4
492.3   Found.   " 56.2, " 6.5, " 11.6

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dimethyl Ester —0.5 gm. of benzoyltetrapeptide was suspended in methanol and esterified with an excess of an ethereal solution of diazomethane. The residue obtained on evaporation was transferred to the filter with ethyl acetate-ether and recrystallized from ethyl acetate. Needles; m.p., 178°; yield, 0.4 gm.

The compound was also more simply prepared by the hydrogenation of the carbobenzoxytetrapeptide dimethyl ester in the presence of 1 mole of methyl alcoholic hydrogen chloride, followed by the benzoylation of the free ester in the usual manner.

$C_{25}H_{36}O_8N_4$ (520.3). Calculated, N 10.8; found, N 10.7

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dihydrazide— 3.6 gm. of the above dimethyl ester were dissolved in hot absolute alcohol, 0.9 cc. of the hydrazine hydrate was added, and the solution allowed to stand overnight at room temperature. The

spongy mass which separated out was washed well with alcohol. Yield, 3.5 gm.; m.p., 253° after recrystallization from alcohol.

$C_{23}H_{26}O_6N_8$ (520.3).   Calculated, N 21.5; found, N 21.1

Repeated recrystallizations did not improve the analysis.

Benzoylglycyl-l-Alanyl-l-Leucyl Derivative of 1-Amino-1 , 3-Di-carbobenzoxypropane—3.3 gm. of the above dihydrazide were dissolved in 25 cc. of water and 12.7 cc. of N hydrochloric acid, the solution was cooled to 0°, and to it was added within 1 minute an aqueous solution of 0.95 gm. of sodium nitrite. The precipitate was separated from the supernatant liquid and washed with cold water containing 6 cc. of N hydrochloric acid, treated with a small volume of ether, filtered, washed with water and ether, and dried well over phosphorus pentoxide in a desiccator. Yield of diazide, 2.4 gm. The dry azide was added in small portions within 3 to 4 minutes to a solution of 20 cc. of xylene and 5 cc. of benzyl alcohol which had been warmed to 90°. After completion of the nitrogenation, the solution was heated for 5 minutes at the boiling point of xylene. On cooling, a spongy mass separated out, the precipitation being made more complete by the addition of ether. Yield, 1.7 gm.; m.p., 201° after recrystallization from 50 per cent acetic acid and washing with ethyl acetate.

$C_{37}H_{46}O_8N_6$.   Calculated.   C 63.2, H 6.6, N 12.0
702.4   Found.   " 63.4, " 6.7, " 12.1

Hydrogenation and Splitting of the Dicarbobenzoxy Compound —1.4 gm. of the dicarbobenzoxy compound were suspended in methanol and 3 cc. of 2 N hydrochloric acid, and hydrogenated in the presence of 0.5 gm. of fresh palladium black. The hydrogenation was completed in about ½ hour and the filtered solution was evaporated to dryness at 30–35°, the residue dissolved in a small volume of water, the solution filtered from traces of undissolved material, neutralized to litmus with sodium hydroxide, and added to a hot saturated aqueous solution of 0.6 gm. of dimethone. The mixture was kept at 100° for about 5 minutes, the unchanged dimethone removed by ether extraction, and the aqueous solution concentrated *in vacuo* at 35° to 4 to 5 cc. On standing overnight, crystals of benzoylglycyl-*l*-alanyl-*l*-leucylamide separated out, which were contaminated with a slight amount of

sirup. They were filtered off, washed with ether, and recrystallized from methanol-water.   Yield, 0.4 gm.; m.p., 186°.

$C_{18}H_{26}O_4N_4$.   Calculated.   C 59.6, H 7.2, N 15.5
362.2   Found.   " 59.8, " 7.3, " 15.3

The mother liquor was made slightly alkaline with sodium hydroxide and acidified slightly with acetic acid. On standing overnight, plates of the dimethone derivative of β-aminopropionaldehyde (0.3 gm.) separated out which, after recrystallization from alcohol, melted at 208°. The mixed melting point with the dimethone obtained from the degradation product of benzoylglutamic acid was 208°.

$C_{19}H_{27}O_3N$ (317.2).   Calculated, N 4.4; found, N 4.6

Benzoylglycyl-l-Alanyl-l-Leucyl Hydrazide—1 gm. of the amide obtained by the above degradation was refluxed for 2 hours in 3 cc. of ethanol and 0.5 cc. of hydrazine hydrate, and then heated for 1.2 hours without refluxing; whereupon ammonia was liberated. On standing overnight, 0.45 gm. of the hydrazide separated out in needles bunched in spheres. M.p., 230° after recrystallization from ethanol.

$C_{18}H_{27}O_4N_5$ (377.2).   Calculated, N 18.6; found, N 18.3

Benzoylglycyl-l-Alanyl Compound of 1-Amino-1-Carbobenzoxy-amino-3-Methylbutane—2.5 gm. of the above hydrazide were dissolved with heating in 7 cc. of glacial acetic acid and 40 cc. of water, the solution was cooled to 0°, and to it was added dropwise an ice-cold aqueous solution of 0.5 gm. of sodium nitrite. After about 10 minutes at 0°, the azide was taken up in about 70 cc. of ethyl acetate, the solution washed repeatedly with ice-cold water, bicarbonate, and again water, and dried over sodium sulfate. To the solution 6 cc. of benzyl alcohol were added. The ethyl acetate was removed *in vacuo* at 35–40°, and then the residue was heated to 80° for ½ hour. On cooling, the urethane separated out in fine needles, which were redissolved by adding benzene and heating. On cooling, 1.1 gm. of substance formed; from the mother liquor 0.2 gm. was obtained. Recrystallization from glacial acetic acid-water gave long needles with a melting point of 212°.

$C_{25}H_{32}O_5N_4$ (468.3).   Calculated, N 12.0; found, N 11.8

Hydrogenation and Splitting of the Carbobenzoxy Compound —1.1 gm. of the carbobenzoxy compound were suspended in methanol which contained 1.1 moles of hydrogen chloride, and hydrogenated in the usual manner. The residue obtained on evaporation *in vacuo* at 35° was dissolved in 10 cc. of water, and the solution heated to boiling in a bath at 125° for 5 to 8 minutes. The distillate was collected in a receiving flask which contained 0.4 gm. of *p*-nitrophenylhydrazine in 10 cc. of 50 per cent acetic acid. In the receiving flask 0.35 gm. of the nitrophenylhydrazone of isovaleraldehyde separated out. After recrystallization it melted at 113°.

$C_{11}H_{15}O_2N_3$ (221.1).   Calculated, N 19.0; found, N 19.3

On allowing the filtered aqueous solution to stand in the ice box, 0.4 gm. of benzoylglycyl-*l*-alanineamide was obtained. After recrystallization from a small volume of methanol, the melting point was 192°.

$C_{12}H_{15}O_3N_3$.   Calculated.   C 57.8, H 6.1, N 16.9
249.1   Found.   " 57.7, " 5.9, " 16.9

Benzoylglycyl-l-Alanine Hydrazide—0.5 gm. of the above benzoylglycyl-*l*-alanineamide was refluxed for 2 hours in 5 cc. of alcohol and 0.4 gm. of hydrazine hydrate, and then heated without refluxing for $\frac{1}{2}$ hour on the steam bath, whereupon ammonia was liberated. On cooling, 0.4 gm. of crystals separated out, which were recrystallized from alcohol. M.p., 212°.

$C_{12}H_{16}O_3N_4$ (264.1).   Calculated, N 21.2; found, N 21.1

Benzoylglycyl Derivative of 1-Amino-1-Carbobenzoxyaminoethane —2.2 gm. of the preceding hydrazide were dissolved in 80 cc. of hot water and 3 cc. of glacial acetic acid, the solution was cooled to 0°, and an aqueous solution of 0.7 gm. of sodium nitrite added dropwise. On scratching, crystals (needles) of the azide were obtained. These were filtered off after $\frac{1}{4}$ hour, washed, and dried on a porous plate in a desiccator over phosphorus pentoxide. Yield, 2 gm. of azide. This was added in small portions (within 10 minutes) to a mixture of 15 cc. of xylene and 5 cc. of benzyl alcohol which had been heated in a bath at 150°. After the nitrogen generation had stopped, ether was added and the spongy

precipitate dissolved in 5 cc. of hot acetic acid. Water was added to cloudiness, and on slow cooling 0.35 gm. of needle-like crystals, with a melting point of 207°, was obtained.

$C_{19}H_{21}O_4N_3$ (355.2). Calculated, N 11.8; found, N 12.1

Hydrogenation and Splitting of the Carbobenzoxy Compound—
0.3 gm. of the carbobenzoxy compound was dissolved in methanol and 0.1 cc. of 10 per cent hydrochloric acid, and catalytically hydrogenated in the usual way. After completion of the hydrogenation, the solution was evaporated down *in vacuo* at 35°, the residue dissolved in about 8 cc. of water, and the solution filtered and heated to boiling for 5 minutes. The vapors were led into an ice-cooled receiving flask which contained a saturated aqueous solution of 0.3 gm. of dimethone. On standing overnight, 0.2 gm. of ethylidene dimethone (m.p., 143°) separated out in the receiving flask. The mixed melting point with the acetaldehyde dimethone obtained with paraldehyde (12) was 143°.

$C_{18}H_{26}O_4$. Calculated. C 70.5, H 8.5
306.2 Found. " 70.4, " 8.3

The original aqueous solution was concentrated *in vacuo* to 2 cc. On standing overnight, crystals (0.1 gm.) of hippurylamide separated out, which had a melting point of 185° after recrystallization from water. The mixed melting point with hippurylamide prepared according to Fischer (13) showed no depression.

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QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

I. QUANTITATIVE FORMOL TITRATION BY MEANS OF THE GLASS ELECTRODE *

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A considerable number of the analytical procedures which have 'been proposed for the quantitative determination of amino acids have been found to be generally applicable to the analysis of amino acids in plant extracts, protein hydrolysates, and clinical fluids, where extreme accuracy is unimportant or unattainable. However, the methods in common use are notably unsatisfactory for the precise analysis of amino acids. Often the results are only semiquantitative because of interfering side reactions, uncertain color changes of indicators at the end-point of titrations, or other uncontrollable factors.

The present work of the authors has been predicated on the assumption that none of the older methods[1] which has been de-

[1] The methods referred to here are the Van Slyke nitrous acid (1), the Sörensen formol titration (2), the Foreman alcohol titration (3), the Linderstrom-Lang acetone titration (4), the Folin β-naphthoquinone sulfonate (5), and the Harding-MacLean ninhydrin (6) procedures. The Ashmarin modified formol titration (7), the Halford molecular boiling point rise (8), and the specific rotation methods were not considered because of their uncertain reliability or limited applicability. The determination of the purity of amino acids by recrystallization to constant solubilities (9), conductivities (10), or heats of combustion (11) should be dependable, although laborious, procedures.

vised for the analysis of amino acids can be relied upon for the attainment of truly quantitative results. It seems probable, as pointed out by Levy (12) for the formol titration method, that about ±0.5 per cent is the limit of accuracy and precision for these procedures.

It has been a common practise to determine the purity of amino acids and peptides by a Kjeldahl or Dumas analysis. While it has been shown that total nitrogen may be estimated by these methods with an error of only 0.1 to 0.2 per cent (13), analyses of this precision indicate less accurately the purity of the material when nitrogenous impurities are present.[2]

In a recent paper, Nadeau and Branchen (14) described a new method by which amino acids in glacial acetic acid are titrated with perchloric acid by means of crystal violet indicator or the chloranil electrode. Results of high precision were obtained in the direct colorimetric titration of glycine with crystal violet indicator and weight burettes. However, deviations of considerably greater magnitude between duplicate determinations as well as between the colorimetric values and those found by micro-Dumas analyses, were reported for ten additional amino acids.

The present method was elaborated because of the need for a procedure by which the purity of amino acids, required for the physicochemical investigations under way in this Laboratory, could be determined with quantitative accuracy. As the result of the authors' investigations it has been found that amino acids in aqueous-formaldehyde solution may be titrated with standard alkali by means of the glass electrode with both a precision and accuracy of ±0.1 per cent.

EXPERIMENTAL

Materials—The amino acids and some of the peptides were highly purified samples prepared in this Laboratory. The peptides, obtained from Hoffmann-La Roche, Inc., were used without further purification.

The solutions of carbonate-free sodium hydroxide were standardized against acid potassium phthalate and Bureau of Standards

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[2] If the analytical error from the determination of the nitrogen content of glycylglycine by the Kjeldahl or Dumas method were 0.1 per cent, 1.26 per cent of glycine could be present and escape detection.

benzoic acid with the use of carefully calibrated instruments throughout.

A 37.5 per cent formaldehyde solution of purest commercial grade, treated with basic magnesium carbonate to remove formic acid, was used in all of the experiments. Dearing (15) has stated that formaldehyde may be maintained permanently neutral by treatment with basic magnesium carbonate. In the present experiments, there was no visible pink color when 12 ml. of the treated 37.5 per cent formaldehyde solution were filtered, diluted to 50 ml., and tested with 1 ml. of phenolphthalein solution. However, a glass electrode determination showed that this solution was slightly alkaline (pH 7.69) owing, presumably, to dissolved basic magnesium carbonate. The special purification procedures employed by Euler and Euler (16) and Levy (17) were not used because the formaldehyde solutions prepared by these authors were slightly acid owing, probably, to formic acid.

Apparatus—The glass electrode apparatus, described by Robertson (18), was used with a few modifications. A silver-silver chloride electrode, prepared essentially by the method of MacInnes and Beattie (19), replaced Robertson's calomel electrode (C_1). The silver-silver chloride electrode was connected by a 1 N potassium chloride bridge, with liquid junction, to a reference solution of 0.1 N hydrochloric acid in the glass electrode bulb.

The electrodes, made from No. 015 Corning glass, consisted of extremely thin glass bulbs with an average resistance of 0.15 megohms. Resistances were determined by comparison of the glass electrode potentials required to produce a given deflection on the galvanometer scale with the potential needed to give the same deflection with a standard, approximately 2 megohm resistance. A maximum change of ±2 millivolts was observed during the life of the electrodes (2 or 3 months). Before each titration in which pH values were determined, the glass electrode was standardized against buffers of known pH determined with the quinhydrone electrode to an accuracy of 0.01 unit. It was shown that the glass electrode acted theoretically as a hydrogen electrode, since the apparent potential interval per pH unit between 2 and 8 was 0.0587 at 23°.

Potentiometric Titration—To carry out a titration, approximately 0.2 to 0.4 gm. of the amino acid, dried for 3 hours at 80°

and 10 mm. in a modified Abderhalden drier, was dissolved in 38 ml. of distilled water.   12 ml. of 37.5 per cent formaldehyde, which had been treated with basic magnesium carbonate and filtered, were added.   In the case of alanine, norleucine, phenylalanine, and α-aminobutyric acid 45 ml. of distilled water and 20 ml. of formaldehyde solution were used.   The motor stirrer was started and stirring continued throughout the titration.   About 1 ml. less than the calculated volume of an approximately 0.3 N standard solution of sodium hydroxide was added slowly over a period of about 10 minutes.   The potential of the system was measured after about 1 minute.   Approximately 0.2 ml. increments of standard base were added, and after 4 minute intervals to permit depolarization of the glass electrode, the voltages were read.   Titrations were carried from 0.1 to 1.0 ml. beyond the equivalence point, depending on the magnitude of the voltage change per ml. of base.   The end-points of the titrations were determined from a plot of $\Delta E/\Delta V$ against V ($\Delta E/\Delta V$ signifies the change in voltage per unit change in volume of base).

For the attainment of smooth curves it was found necessary to avoid adding any water during the titration, to add the base directly to the solution away from the stirrer, and to measure the volumes of base with an error $\leq$ 0.05 per cent.

DISCUSSION

Complete titration data and $\Delta E/\Delta V$ values from the analysis of three samples of one amino acid, glycine, are given in Table I.   In order to conserve space detailed analytical information on six additional amino acids and nine peptides has been omitted. In the latter cases summaries of the experimental results are listed in Tables II and III.

The precision obtained in the titration of amino acids is indicated by the differential plots, shown in Fig. 1, of the experimental data from the analysis of three glycine samples.   In these determinations 30.73, 30.57, and 33.50 ml. were the corrected volumes of 0.2963 N sodium hydroxide solution required for the titration of glycine Samples I, II, and III respectively.   The percentages of the theoretical equivalent weights calculated from these corrected end-point volumes of standard base were 99.59, 99.74, and 99.70. The mean deviation for these experimental values was found to

be ± 0.06 per cent. Mean deviations, ranging from ± 0.03 to ± 0.10 per cent, and an average mean deviation of ± 0.05 per cent, were calculated from the analytical data for all of the amino acid determinations given in Table II.

The deductions of Eastman (20) and Roller (21) seem to indicate the degree of precision which can be attained in the formol titration of amino acids. These authors have shown that the condition of an inflection in the titration of a weak acid by a strong base is that $cK_A > 27K_W$, where K_A is the apparent ioniza-

TABLE I

Titration Data and $\Delta E/\Delta V$ Values from Analysis of Glycine

Sample I			Sample II			Sample III		
E.M.F.	Volume of base	$\dfrac{\Delta E \times 10^5}{\Delta V \times 10^2}$	E.M.F.	Volume of base	$\dfrac{\Delta E \times 10^5}{\Delta V \times 10^2}$	E.M.F.	Volume of base	$\dfrac{\Delta E \times 10^6}{\Delta V \times 10^2}$
volt	*ml.*		*volt*	*ml.*		*volt*	*ml.*	
0.4035	29.67		0.4172	29.84		0.4161	33.62	
0.4116	29.88	38.5	0.4271	30.06	45.0	0 4317	33.00	41.1
0.4221	30.11	45.7	0.4408	30.25	72.1	0.4454	33.20	68.5
0.4349	30.31	64.0	0.4649	30.46	115.0	0.4646	33.40	96.5
0.4528	30.52	85.5	0.4796	30.59	112.0	0.4755	33.50	108.0
0.4640	30.62	112.0	0.4919	30.74	80.2	0.4932	33.70	88.5
0.4671	30.72	121.0	0.5052	30.96	60.5	0.5141	34.10	52.5
0.4855	30.82	94.0						
0.5014	31.03	75.7						

The weights of samples taken were: Sample I, 0.6858 gm., Sample II, 0.6816 gm., and Sample III, 0.7471 gm. Time intervals of 4 minutes, 0.2963 N sodium hydroxide, and a temperature of $22°$ were used in all of the titrations. The expression, $\Delta E/\Delta V$, signifies the change in voltage per unit change in volume of base.

tion constant of the acid, K_W the ion product constant of water, and c defined by the equation $1/c = 1/a + 1/b$. In the latter equation a and b are the initial concentrations of acid and base respectively. It was shown further that an inflection point should appear, and that a titer deviation of 0.03 per cent between the stoichiometric end-point and the inflection point values should result, when $c = 0.1$ and K_A is 10^{-10}. Since, in the present investigations, c was approximately 0.1 and K_A (in aqueous formaldehyde solution) of the amino acid with the smallest apparent

ionization constant[3] was approximately 10^{-7}, in all cases the inherent analytical error would seem to be <0.03 per cent.

While the precision of the results from the titration of the peptides, glycylglycine (Sample I) and *dl*-leucylglycine, was compar-

<div align="center">

TABLE II

Summary of Experimental Data from Analysis of Amino Acids

</div>

Amino acid	Percentage of theoretical equivalent weight	Amino acid	Percentage of theoretical equivalent weight
Glycine	99.70	*dl*-Alanine (Sample I)	99.85
	99.66		99.66
	99.59	" (" II)	100.53
dl-Serine (Sample I)*	99.00		100.63
	99.06	" (" III)	99.84
" (" II)†	99.97		99.76
	99.91	*dl*-Phenylalanine	99.71
" (" III)‡	99.23		99.57
	99.09	*d*-Glutamic acid in presence of glycine¶	100.10
" (" IV)§	99.47		100.20
" (" V)‖	100.07	*dl*-α-Aminobutyric acid**	100.64
	100.21		
dl-Leucine	100.68		
	100.78		

* Prepared by Frank J. Ross from ethoxyacetaldehyde by the method of Dunn, Redemann, and Smith (25).

† Prepared by recrystallization of Sample I.

‡ Prepared by Palmer Stoddard. The product was dried at 55° for 3 days.

§ Prepared by drying Sample III in an Abderhalden drier for 3 hours at 80° and 10 mm. in the absence of drying agent.

‖ Prepared by drying Sample III in the Abderhalden drier for 3 hours at 80° and 10 mm. in the presence of anhydrite (anhydrous calcium sulfate) (26).

¶ 0.3000 and 0.4000 samples of *d*-glutamic acid respectively, each in the presence of 10.00 gm. of glycine, were titrated in aqueous solution.

** Prepared by Nathaniel L. Smith.

able to that obtained with amino acids, in general the peptide analyses were unsatisfactory. Although the peptides examined

[3] The apparent acid dissociation constants in aqueous formaldehyde of a series of amino acids and peptides are to be reported in a forthcoming paper.

in this study were known to be purified products, it appears that they were not of high purity or homogeneity. However, only a limited number of peptide analyses could be made, owing to the small amount of available material.

The glass electrode analysis of amino acids and peptides appears to be an accurate measure of purity when only inert contaminants

TABLE III

Summary of Experimental Data from Analysis of Peptides

Peptide	Percentage of theoretical equivalent weight	Peptide	Percentage of theoretical equivalent weight
Glycylglycine (Sample I)*	98.72	Glycyl-*dl*-leucine†	96.65
	98.87		97.66
	98.95	*d*-Leucylglycine‖	95.87
	98.68		97.72
" (" II)†	101.81	*dl*-Norleucylglycine¶	96.07
	100.83		96.34
	100.98	*dl*-Alanylglycine†	98.55
" (" III)‡	96.93		99.04
	97.72	*l*-Leucyl-*l*-tyrosine†	97.97
Diglycylglycine†	96.58		95.32
Triglycylglycine§	96.19		
dl-Leucylglycine†	97.01		
	97.07		

* Prepared by Sidney Fox from diketopiperazine.
† Hoffmann-La Roche product.
‡ Prepared by Thorpe Deakers from chloroacetyl chloride and glycine by the method of Dunn, Butler, and Deakers (27).
§ Prepared by Thorpe Deakers from chloroacetyl chloride and diglycylglycine.
‖ Hoffmann-La Roche product. This material was very hygroscopic.
¶ Prepared by Lee Read from α-bromocaproyl chloride and glycine.

are present. While strongly acidic or basic impurities would destroy the accuracy of the determinations, fortunately such substances may be readily removed by simple purification procedures. When an amino acid is the impurity, quantitative results can be expected from titrations with the glass electrode in those cases in which there is a 10,000-fold (22) difference between the apparent acid dissociation constants of the component substances. The

authors' data on the titration in aqueous solution of *d*-glutamic acid in the presence of glycine illustrate the validity of this principle.

On the other hand, peptide titrations would be subject to a different interpretation.   Even in aqueous formaldehyde solution there is not more than a 100-fold difference between the apparent
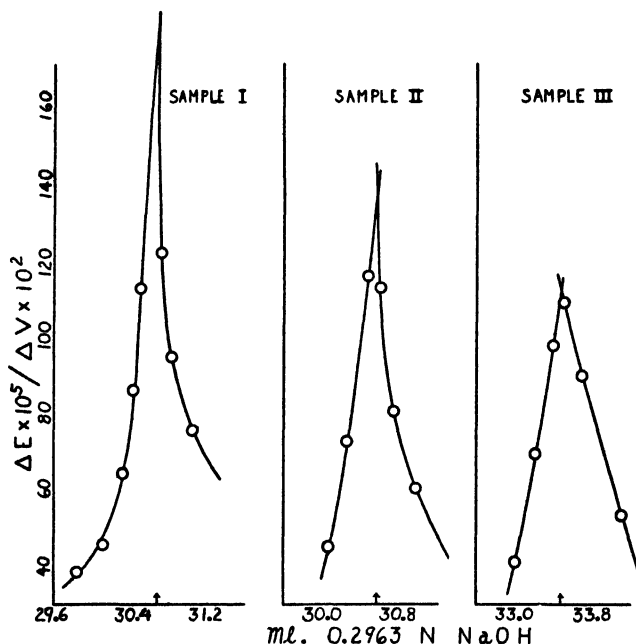
FIG. 1. Curves showing the volumes of standard base required to titrate glycine Samples I, II, and III respectively.   The volume of standard base at the equivalence point is indicated in each case by an arrow extending upward from the base-line.

acid dissociation constants of a peptide and its amino acid constituents.   While it could be assumed that the end-point would be sharp in titrating a peptide which contained a small amount of an amino acid impurity, the equivalents of base at the inflection point should be the sum of the equivalents of peptide and amino acid in the sample.   Furthermore, the number of equivalents of base at the inflection point would be greater than that taken for

analysis, when the latter is calculated on the assumption that the sample was pure peptide.

An experiment was performed to test the validity of these conclusions.[4] In the titration of a mixture containing approximately 96 per cent of glycylglycine and 4 per cent of glycine, it was found that the $\Delta E/\Delta V$ values rose sharply to a maximum during the addition of three 0.03 ml. portions of standard base at the inflection point. Hence, the total volume of base, 19.66 ml. at the equivalence point, could be estimated precisely. The equivalents of base used in the titration exceeded by 3.0 per cent those calculated on the assumption that the sample was wholly glycylglycine. However, the number of base equivalents was only 0.63 per cent higher than the sum of the equivalents of the glycylglycine and glycine taken for analysis. This value is considered to be within the limits of the probable experimental error since the latter is the summation of three titration errors.

A minimum error of approximately 1.0 per cent appears to be inherent in many of the titration procedures which have been proposed for the analysis of amino acids and peptides. A common difficulty in Sörensen's classical formol titration procedure (2), published in 1908, and in many later modifications has been the inability to provide end-points which are sharply defined. The precision and accuracy of Sörensen's original method have not been greatly improved by the use of end-points more alkaline than Sörensen's third color stage with phenolphthalein; by the substitution of thymolphthalein (23) and other indicators for phenolphthalein; by the employment of media containing ethyl alcohol and other solvents in addition to water and formaldehyde; by the use of varying concentrations of formaldehyde; or by the determination of equivalence points with the hydrogen electrode (17) or conductive apparatus (24).

The Linderstrom-Lang (4) and the Nadeau and Branchen (14) titrimetric methods seem to be more satisfactory than other procedures. The latter method was discussed earlier in the present paper. Linderstrom-Lang titrated amino acids in concentrated

[4] A mixture, containing 0.005361 equivalent of glycylglycine (0.7169 gm. of 98.9 per cent purity) and 0.000420 equivalent of glycine (0.0361 gm. of 99.8 per cent purity), was titrated in aqueous formaldehyde solution with 0.2963 N sodium hydroxide solution with the use of the glass electrode.

aqueous acetone with 0.1 N 90 per cent alcoholic hydrochloric acid with the use of naphthyl red as indicator. Under optimum conditions of indicator color and acetone concentration the average experimental error was approximately 0.5 per cent.

The conditions which Levy (12) deduced from theoretical considerations to be of fundamental importance in the formol titration of amino acids were adopted in the present work. Within practical limits, the amino acid solutions and the diluting fluids were of the highest possible concentration. The amino acid solutions were at approximately pH 6 prior to the addition of formaldehyde, the end-point of the titration was at about pH 9, no correction was made for the volume of standard base required to bring the aqueous formaldehyde solution to the end-point pH of the amino acid titration, and the concentration of the formaldehyde solution at the end of the titration was between 6 and 9 per cent.

According to Levy's calculations (12), the intrinsic error is 0.5 per cent when monoaminomonocarboxylic acids are titrated under the most favorable conditions. In the present studies it has been demonstrated that the inherent error is ±0.1 per cent. A plausible explanation for this disagreement would seem to be the probable uncertainties in the evaluation of the equation which Levy set up as an expression for the error in the formol titration at the stoichiometric point.

SUMMARY

A method for the quantitative determination of monoamino-monocarboxylic acids, consisting essentially of a formol titration by means of the glass electrode, has been described. The precision attainable in the analysis of amino acids and peptides was found to be ±0.1 per cent. The method was found to have an inherent accuracy of ±0.1 per cent. The probable effects of different types of impurities on the accuracy of the method were discussed.

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THE ISOELECTRIC POINT OF ADSORBED HEMO-GLOBIN*

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It is well established that various proteins, as gelatin and albumin, can be adsorbed on various adsorbents so that the protein-coated particle of adsorbent behaves electrophoretically as a particle of protein (Loeb, 1923; Freundlich and Abramson, 1928). Dummett and Bowden (1933) have recently reported, however, that the behavior of adsorbed hemoglobin varies with the adsorbent surface. Thus, when ox hemoglobin was added in sufficient concentration to coat completely particles of quartz, evacuated blood charcoal, and colloidal copper, respectively, the isoelectric point on quartz was 5.82, on charcoal 5.83, and on copper 6.72. They postulate a binding of some of the ionized groups of hemoglobin by the adsorbent surface.

While this interpretation of their results is reasonable, it is not the only one possible. The other possibility is that other substances of lower isoelectric points than hemoglobin might have been present and preferentially adsorbed. Since no one can guarantee that any sample of hemoglobin is 100 per cent pure, a decision between the two possibilities may be reached by observing whether or not the isoelectric point of adsorbed hemoglobin approaches that obtained by the moving boundary method as the purity of the sample of adsorbed hemoglobin is increased. This we have done and find that the isoelectric point of adsorbed hemoglobin does approach that of freely dissolved hemoglobin with increasing purity. This indicates that Dummett and Bowden's results were due to impurities of lower isoelectric point than hemoglobin. Since the impurities may be preferentially adsorbed, a

small percentage of contaminant might suffice to shift the isoelectric point significantly.

Electrophoresis determinations were made in a cylindrical cell of a modified Mattson type (1933) with the technique previously described (White and Monaghan, 1935). Observations were at a level of 0.147 cell diameter below the cell roof, at which level there is no electroosmotic movement. The visual axis was the vertical diameter of the cell. Glass or quartz particles of 1 to 2 μ diameter were suspended in the hemoglobin solutions and their movements observed. A field strength of 9.1 volts per cm. was used; five observations of particle movement for each direction of current were made and the average velocity computed. In the first series of experiments horse hemoglobin was used in three stages of purification with respect to non-hemoglobin protein. A stock solution was prepared as follows. The cells from fresh oxalated horse blood were washed five times with 10 times their volume of 0.9 per cent NaCl solution to free them from plasma proteins, the washed cells laked by adding 10 volumes of distilled water, and the white cells centrifuged down.

A sample of this stock, which contained stroma material, was diluted 100 times with HCl-KCl or NaOH-KCl mixtures of varying pH, the pH being varied by varying the proportion. The concentration was kept at 0.02 M; pH was measured in all cases with the glass electrode after addition of the hemoglobin. The final dilution was thus about 0.03 per cent hemoglobin; this concentration is more than enough to coat glass particles completely, as is shown by the fact that the isoelectric point and mobilities of the particles do not change further in higher concentrations. From the mobilities observed at different values of pH a curve was constructed which crossed 0 mobility at pH 5.50; i.e., the isoelectric point is at pH 5.50.

The above procedure was repeated except that the stock solution was first passed through a Seitz filter, removing part of the stroma material, as evidenced by the finding that ghosts were no longer found in the filtrate. Glass particles suspended in this product were isoelectric at pH 6.26.

The stock solution was first brought to about pH 5.5 by slow

addition of 5 cc. of 0.1 N HCl to 50 cc. of laked cell stock. A precipitate brought about by the mutual aggregation of hemoglobin and stroma material is formed, since at pH 5.5 hemoglobin is on the acid side of its isoelectric point (6.8) and stroma material is on the alkaline side of its isoelectric point (about 3.0) (*cf.* White and Monaghan, 1936). The material is then passed through a Seitz filter, with a resultant more nearly complete removal of stroma material than is effected by filtration without acidification. Glass particles suspended in this filtrate are isoelectric at pH 6.73.

Similar observations were carried out with beef hemoglobin adsorbed on quartz particles. Here the isoelectric point of the preparation filtered without acidification was at pH 6.65 and that of the preparation filtered after acidification (3 cc. of 0.1 N HCl to 50 cc. of 1:10 laked cell stock), *i.e.* the one most nearly free from non-hemoglobin protein, was at pH 6.77.

DISCUSSION

It is seen that as steps are taken to remove more completely the stroma material from a fresh hemoglobin solution, the isoelectric point of the hemoglobin preparation approaches 6.8, that shown by the moving boundary method. It is evident that such contamination with stroma material would not affect determinations based on observations of the movement of the colored hemoglobin column. With the microscopic method, however, it is essential that no protein other than the one under investigation be present, since the contaminant may be adsorbed on the particle with the result that the observed isoelectric point is the resultant of those of the protein under investigation and of contaminant and will vary with the proportion of these adsorbed. This difficulty may also exist with microscopic observation of hemoglobin crystals, since the crystal surface may adsorb non-hemoglobin protein. This presumably accounts for Abramson's (1934) observation that the isoelectric point of hemoglobin crystals is usually less than 6.8.

It follows that the properties of the hemoglobin are not altered by adsorption on quartz or glass, as Dummett and Bowden supposed. Thus the microscopic method, so far as concerns any case at present known, gives the true isoelectric point of a pure

protein. It is granted that cases may subsequently be observed where the electrical behavior of the adsorbed protein may be different from that of the unadsorbed. Practical deductions from this work are that the microscopic method may be used to test a hemoglobin sample for freedom from non-hemoglobin protein, and that in the preparation of pure hemoglobin crystals it should be advantageous to pass the laked blood through an asbestos filter to reduce greatly the content of stroma material.

SUMMARY

The more nearly free a preparation of hemoglobin is from non-hemoglobin protein the more nearly does the isoelectric point approach 6.8, that seen with the moving boundary method. With the microscopic method, where a surface is involved, a protein must be pure in order to get correct values of mobilities and isoelectric point, but the electrical properties of the protein are not changed by adsorption on quartz or glass. These remarks apply to all proteins so far investigated, including hemoglobin, although exceptions may later be discovered.

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HEXOSEMONOPHOSPHATES

GLUCOSE-4-PHOSPHATE

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Several years ago there was proposed[1] a mechanism of alcoholic fermentation in which the first step was assumed to be the formation of a hexosemonophosphate which then underwent cleavage into a triosephosphate and a phosphorus-free triose. Although a search has been made for such a substance, no hexose ester has been described, with the possible exception of the Robison ester, which ferments as rapidly as does glucose under equivalent conditions. An attempt was therefore made to prepare glucose-4-phosphate as being the most interesting of the remaining possible esters.

Tetraacetyl glucose, identical in properties with that described by Helferich and Klein[2] as β-1, 2, 3, 6-tetraacetyl glucose, was prepared by carefully hydrolyzing β-triacetyl 4, 6-benzylidene glucose, partially acetylating the product, and purifying it by crystallization from pyridine. After removing the pyridine and recrystallizing the product, a pure substance was secured. This was phosphorylated in pyridine with phosphorus oxychloride in the usual manner and the deacetylated phosphoric acid ester was isolated and purified as the dibrucine salt. The barium salt was in turn prepared, and from it the sodium salt.

The sodium salt was employed in fermentation experiments, zymin from three sources being used for the enzyme. It was found that not only did the ester not abolish the induction period observed with certain of the zymins, but also its rate of fermentation,

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[1] Raymond, A. L., *Proc. Nat. Acad. Sc.*, **11**, 622 (1925).

[2] Helferich, B., and Klein, W., *Ann. Chem.*, **450**, 219 (1926); **455**, 173 (1927).

even in the presence of hexosediphosphate, was negligible. At low concentrations of the ester the evolution of carbon dioxide was identical with that of the controls, while at high concentrations the ester was inhibiting and less carbon dioxide was observed than from the controls. In no case did the rate of fermentation in the presence of the ester approach that of glucose under equivalent conditions.

This ester therefore cannot be considered as a direct fermentation intermediate, and it remains only to establish that it is really glucose-4-phosphate, as assumed. That it might not have this structure is not improbable in view of the rather abnormal reactions which have been observed for substituents of glucose in position (4). Thus, β-1, 2, 3, 4-tetraacetyl glucose, on treatment with the mildest alkali, rearranges and gives the tetraacetate mentioned above, described by Helferich and Klein[2] as β-1, 2, 3, 6-tetraacetyl glucose. This acetate on tolylsulfonation and conversion through the bromo compound to the β-methyl glucoside also behaves peculiarly, for on treatment with anhydrous alkali it loses not only the acetyl groups but the tolylsulfo group as well, giving an anhydro methyl glucoside.[3] Finally, there must be mentioned the related observations of Levene and Raymond[4] who attempted to prepare xylose-3-phosphate but in every case obtained, by rearrangement, what was apparently xylose-5-phosphate. In view of these observations, confirmation of the structure of the ester described herein seemed desirable.

The optical rotations of the salts of the new ester do not show significant differences from those of other known esters. In contrast to the case of the glucose-6-phosphate, which gives a crystalline phenylhydrazine salt of the osazone with great ease, the present ester gives no osazone under similar conditions. However, by removing only half the barium from the salt, there was isolated a crystalline acid-barium salt of the osazone. On this basis the ester is established as different from both glucose-3- and glucose-6-phosphate. Josephson and Proffe[5] did not report osazone formation on their glucose-5-phosphate, so no comparison with it is possible.

[3] Helferich, B., and Müller, A., *Ber. chem. Ges.*, **63**, 2142 (1930).
[4] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 347 (1933); **107**, 75 (1934).
[5] Josephson, K., and Proffe, S., *Biochem. Z.*, **258**, 147 (1933).

For final evidence, a study was made of the process of glucoside formation of the ester, for which several comparisons are available. Under the same conditions as those used for other phospho esters, glucoside formation was observed at room temperature and at 76°. The data are given in Table I, and by reference[6] it will be seen that the changes are quite unlike those of either glucose-3-phosphate or glucose-6-phosphate. The 5-phosphate has unfortunately not been studied. Studies on several substituted glucoses[7] had shown that when positions (4) and (5) are unsubstituted, the form of the glucoside curves closely resembles that of glucose itself, whereas the present phospho ester is entirely dissimilar. The ester does, however, show some faint resemblance to tetramethyl glucopyranose, as might be expected of glucose substituted in position (4). Thus the evidence to date indicates that the new ester is in fact glucose-4-phosphate, no rearrangement having occurred during its preparation.

<div align="center">EXPERIMENTAL</div>

β-Triacetyl 4, 6-Benzylidene Glucose—Benzylidene glucose was prepared essentially according to the procedure of Zervas[8] and dried without recrystallization. 100 gm. of the crude dry product and 50 gm. of fused powdered sodium acetate were added to 450 cc. of acetic anhydride. The mixture was warmed with stirring until the temperature reached 35° and was then stirred without further warming until the temperature no longer increased. It was heated on the steam bath for 3 hours and over a free flame for 10 minutes, and then cooled and poured onto ice. The product crystallized immediately but was allowed to stand overnight in the ice box for crystallization to become complete. The product was filtered off and sucked as dry as possible. 300 cc. of 95 per cent alcohol were added, and the mixture was digested at 50° for a few minutes and filtered rapidly. Another 300 cc. of alcohol were added and the extraction was repeated. The α form was almost entirely in solution in the two extracts, along with some

[6] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **81**, 279 (1929); **89**, 479 (1930).

[7] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **96**, 449 (1932). Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **97**, 751 (1932).

[8] Zervas, L., *Ber. chem. Ges.*, **64**, 2289 (1931).

of the β form.   The residual insoluble portion was recrystallized
from ethyl acetate and was obtained pure on one crystallization.
The yield was about 50 gm. from 100 gm. of crude benzylidene
glucose.

β-1, 2, 3, 6-Tetraacetyl Glucose—100 gm. of pure β-triacetyl 4,
6-benzylidene glucose were added to a solution of 900 cc. of ace-
tone and of 100 cc. of 0.25 N aqueous hydrochloric acid.   The
mixture was refluxed for 5 hours, cooled, and concentrated under
reduced pressure until two layers formed.   The mixture was
repeatedly extracted with pentane by decantation to remove al-
most all of the benzaldehyde and was then concentrated under
reduced pressure to a syrup.   500 cc. of chloroform were added,
and then enough anhydrous sodium sulfate to combine with all
the water present.   The mixture was filtered and the sodium sul-
fate was twice extracted with boiling chloroform.   The combined
chloroform extracts were dried again with anhydrous sodium
sulfate and concentrated under reduced pressure to a syrup.
This was dissolved in 200 cc. of dry pyridine; 120 cc. of chloroform
were added and the solution was cooled in ice.   32 cc. of acetic
anhydride (1.33 moles per mole of starting material) were added;
the mixture was allowed to remain in the ice-bath for an hour and
then at room temperature overnight.   5 cc. of water were added
and after ½ hour at room temperature, more water.   The product
was extracted with chloroform, and the extracts were washed with
acid to remove pyridine and then twice with water.   They were
dried with anhydrous sodium sulfate and concentrated to a
syrup under reduced pressure.   100 cc. of dry pyridine were added
and the solution was placed in the ice box.   The crystals, which
formed easily and spontaneously, were filtered off, washed with a·
little cold ether, and dried.   By further concentration two more
crops of material were similarly secured.   The entire product was
recrystallized from pyridine, or occasionally from ether containing
about 10 per cent of pyridine.   The material as thus prepared
agreed in properties with that described by Helferich and Klein.[2]
For preparation of the pyridine-free product the above material
was dissolved in chloroform, and the solution was extracted with
acid, washed with water, dried, and concentrated.   The product
crystallized well from ether and agreed in properties with that of
Helferich and Klein.[2]   The yield of pure product is only about 15

gm. from 100 gm. of starting material, but the low yield is due to a considerable extent to the difficulty of purification. A further amount of material is evidently contained in the various solutions, but its recovery is very difficult.

Phosphorylation of β-1, 2, 3, 6-Tetraacetyl Glucose—10 gm. of the pure tetraacetate were dissolved in 20 cc. of pure dry chloroform, cooled, and added to a solution of 3.0 cc. of freshly distilled phosphorus oxychloride in 100 cc. of dry pyridine at −30°. After a few minutes the mixture was transferred to an ice-salt bath and over a period of 2 hours was allowed to reach room temperature as the bath melted. A precipitate, which was present at first, slowly dissolved and after the 1st hour the solution was clear. At the end of the time considerable ice was added and then saturated barium hydroxide until the solution was alkaline to thymolphthalein. On standing, the mixture became less alkaline and more barium hydroxide was added until alkalinity was maintained for 20 minutes. The mixture was then filtered with charcoal and concentrated under reduced pressure until all pyridine was removed. Sulfuric acid was added to remove the barium quantitatively, and the mixture was centrifuged. A warm solution of brucine in methyl alcohol was added to pH 7.4 to 7.6 (100 gm. of brucine were required), and the mixture was filtered with charcoal. The solution was concentrated under reduced pressure to a volume of about 100 cc. and an equal volume of acetone was added. On cooling and stirring, a product crystallized. This was filtered off, dissolved in warm water containing acetone, filtered with charcoal, and again concentrated under reduced pressure to a volume of about 100 cc. On addition of an equal volume of acetone, cooling, and stirring, the pure product crystallized. It was washed successively with acetone, ether, and finally pentane, and was then dried over calcium chloride and potassium hydroxide under greatly reduced pressure. The yield was 9.7 gm. or 32 per cent. The composition corresponded to the dibrucine salt of a hexosephosphoric ester.

7.830 mg. substance: 0.382 cc. N (at 32° and 766.5 mm.)
7.170 "   "   : 14.100 mg. ammonium phosphomolybdate (Pregl)
$C_{52}H_{66}O_{17}N_4P$. Calculated. N 5.35, P 2.96
.   1048.6   Found.   " 5.52, " 2.85

The rotation was as follows:

$$[\alpha]_D^{23} = \frac{-1.81 \times 100}{2 \times 1.996} = -45.3° \text{ (in pyridine)}$$

$$[\alpha]_D^{23} = \frac{-0.39 \times 100}{2 \times 1.998} = -9.8° \text{ (in 20 per cent alcohol; 15 minutes after dissolving and unchanged after 2 hours)}$$

Barium Salt of the New Phosphoric Ester—8.0 gm. of the pure brucine salt of the ester were dissolved in warm water and a saturated solution of barium hydroxide was added until the solution was alkaline to phenolphthalein. On stirring, the brucine crystallized out and was removed by extraction with chloroform. The aqueous solution was concentrated under reduced pressure to a small volume and the barium salt precipitated with an equal volume of alcohol. The product was centrifuged off, suspended in absolute alcohol, and recentrifuged. This procedure was repeated, acetone, acetone plus ether, and ether being used successively. The final product was dried over phosphorus pentoxide under greatly reduced pressure. The yield was 2.7 gm. or 89 per cent. Its composition corresponded fairly well to the barium salt of a hexosephosphoric ester.

8.290 mg. substance: 41.810 mg. ammonium phosphomolybdate (Pregl)
33.300 "   "   : 20.200 " BaSO₄
              C₆H₁₁O₉PBa.   Calculated.   P 7.84, Ba 34.74
              395.5   Found.   " 7.32, " 35.69

The product apparently contained a small amount of barium phosphate, as the aqueous solutions were turbid.

Osazone Formation—From a pure sample of the barium salt the barium was quantitatively removed with sulfuric acid. 4 moles of phenylhydrazine (in glacial acetic acid) were added per mole of ester and the mixture was heated on the steam bath. After some time a dark tarry product resulted, but in small amount and crystallization was not achieved.

The above experiment was repeated, but with the single exception that half the barium was removed by adding the calculated amount of sulfuric acid. The osazone formed within a few minutes, and the mixture was allowed to stand in the ice box. The osazone was filtered off, washed with a little methyl alcohol,

and dried. It consisted of a mass of lemon-yellow plates. The composition corresponded approximately to an acid-barium salt of a phosphohexosazone.

4.610 mg. substance: 17.720 mg. ammonium phosphomolybdate (Pregl)
5.600 "   "   : 0.500 cc. N (26° and 759 mm.)
20.601 "   "   : 6.670 mg. BaSO₄
Calculated barium-free
        $C_{18}H_{23}O_7N_4P$. Calculated. N 12.79, P 7.08
        438.3   Found.   " 12.56, " 6.87

Glycoside Formation—The same concentrations and procedures were used as in the case of the other phospho esters which have

TABLE I

Specific Rotation during Glycoside Formation

Time		Specific rotation	
		28°	76°
hrs.	*min.*	*degrees*	*degrees*
0	5	20.6	
0	11	22.1	
0	22	23.9	
0	40	25.4	
1	0		25.0
1	30	25.4	
3	0		20.0
3	30	25.4	
7	0		17.3
26	0	28.8	
72	0	34.8	
124	0	34.6	

been studied.[6] Glycoside formation was observed at room temperature (average 28°) and in sealed tubes at 76° (in a boiling carbon tetrachloride bath). In the latter case tests of inorganic phosphate were made which showed that the hydrolysis of the phospho group was not significant during the period employed (24 hours), although a small amount of inorganic phosphate was present at the start. The results are presented in Table I.

Comparison with the earlier data for glucose-3- and glucose-6-phosphate[6] shows the new ester to behave in entirely different

fashion. The glucose-5-phosphate of Josephson has not been similarly studied. A further comparison[7] shows the new phosphate to be somewhat related in behavior to tetramethyl glucopyranose in which position (4) is definitely blocked.

Fermentation Experiments—The barium salt of the ester was converted to the sodium salt by adding the exact equivalent of sulfuric acid followed by sodium hydroxide to pH 6.4. This solution was used in fermentation experiments in which zymin from three different sources was employed. The ester was used at concentrations of 0.005, 0.02, and 0.06 M in the fermentation mixtures, in the presence and absence of glucose and of hexosediphosphate. The ester did not significantly reduce the induction period which was observed with two of the zymins in the absence of hexosediphosphate. At concentrations of 0.005 and 0.02 M the observed fermentation in the presence of the ester was identical with that of the control, while at a concentration of 0.06 M the ester was inhibiting and the fermentation was less than that of the control. In no case did the fermentation rate of the ester approach that of glucose-inorganic phosphate mixtures with the same zymin.

THE EFFECTS OF INHALATION OF CARBON DIOXIDE ON THE CARBON DIOXIDE CAPACITY OF ARTERIAL BLOOD

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. Studies of the effects of inhalation of CO_2 on the arterial blood have not yielded uniform results, for a decreased CO_2 capacity has been noted by some observers (Shaw and Messer, 1932) and an increased one by others (Henderson and Haggard, 1918). It is the purpose of the present investigation to examine this discrepancy.

Methods

Observations were made on human subjects and on dogs and cats exposed to an atmosphere consisting of a 5 to 8 per cent mixture of CO_2 in air. The inhalation of CO_2 took place in a chamber large enough to admit two patients and three observers.[1] The chamber was equipped with an apparatus for conditioning the gas mixtures respired and a device for recording the concentration of CO_2 and O_2 respectively on alternate minutes (Bullowa and Lubin, 1931).

The samples of blood were drawn either from the brachial or femoral artery, before during, and, in some instances, after exposure to CO_2. These samples were divided into two portions. The first was analyzed for pH (DuBois, 1932), lactic acid (Friedemann, Cotonio, and Shaffer, 1927), CO_2 and O_2 content and capacity (Van Slyke and Neill, 1924). The equilibration of the blood samples with CO_2 and O_2 for the determination of the CO_2 and O_2

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[1] The funds for the construction of this chamber were supplied by a grant from the Research Fund of Yale University.

capacity was made as previously described (Himwich and Rose, 1929). The second portion of the blood was delivered into a stoppered test-tube and permitted to clot. The serum was then analyzed in different experiments for total base (Stadie and Ross, 1925), bicarbonate, chloride (Eisenman, 1929), proteins by the Kjeldahl method, total solids, and specific gravity (Barbour and Hamilton, 1926).

Results

Seven experiments were made on the human subjects, one each on two normal controls, and five on four patients with schizophrenia. In every instance of an exposure to CO_2 of half an hour's duration, the CO_2 capacity of the blood fell. The decreases for the seven experiments were 5.37, 4.77, 4.32, 3.02, 1.75, 1.30, and 0.54 volumes per cent. This occurred despite a rise in CO_2 content. The O_2 capacity increased significantly once, five times it rose, and once it fell within the error of the method (1 volume per cent). The pH of the blood diminished as a result of the inhalation of CO_2 but the concentration of lactic acid remained unchanged in each of three experiments. Since the results of all seven experiments are similar (Gildea, Himwich, Hubbard, and Fazikas, 1935), a typical example is presented in Table I.

On seven occasions four dogs were exposed to the CO_2 mixtures for a longer time than were the human subjects. Such a longer period of inhalation disclosed a diphasic variation. In addition to an early decrease of the CO_2 capacity in four of six observations, there was a later rise. Dogs A and B, like the human subjects, exhibited a fall in CO_2 capacity in blood drawn within the first half hour after inhalation of CO_2 had begun. Dog C reacted similarly on two of three occasions. In the four observations of Dogs A, B, and C which disclosed this fall, the decreases were 7.43, 2.43, 1.66, and 0.98 volumes per cent. However, in a third experiment on Dog C and one on Dog D, the CO_2 capacity rose but did so to a lesser extent than did the content; i.e., the content increased 6.9 and 2.6 volumes per cent, while the capacity was raised 1.2 and 1.1 volumes per cent respectively. In an observation on Dog C made at 55 minutes, as well as on all other observations made at periods longer than that one, the CO_2 capacity was found to be raised.

TABLE I

Effects of Inhalation of Carbon Dioxide on Blood Constituents

Subject	Blood sample	Time	Atmosphere	CO₂ tension	CO₂ content	CO₂ capacity	O₂ content	O₂ capacity	pH	Lactic acid	Total solids	Protein	CO₂	Cl	Acid protein	Total acid	Total base
		min.	per cent CO₂	mm. Hg	vol. per cent	vol. per cent	vol. per cent	vol. per cent		mg. per cent	gm. per cc.	per cent	m.-eq.	m.-eq.	m.-eq	m.-eq.	m.-eq.
Human, J	A₁	0	Room	35	45.03	48.84	18.7	21.83	7.41	16							
	A₂	30	5.4	55	49.22	43.47	19.15	21.89	7.25	13							
Dog B	A₁	0	Room	36	44.21	44.06	19.23	22.66	7.42	13	0.0861	6.7	26.4	110	16.0	152.4	159
	A₂	20	8.0	57	50.59	41.63	19.52	22.27	7.27	16	0.0871	6.8	29.4	110	15.3	154.7	161
	A₃	94	6.1	47	52.66	47.40	18.22	20.21	7.39	17	0.0850	6.5	30.5	110	15.3	155.8	160

Determination of the pH of the whole blood revealed a fall which was greatest with the largest decrease of CO_2 capacity. A definite if only partial recovery was observed on the occasions on which the CO_2 capacity rose. The changes of CO_2 capacity were not associated with variations of the concentration of lactic acid of the whole blood (two experiments on human and three on canine subjects) nor with those of the acids or bases of the serum. There was no significant change of total base (five experiments), chlorides (seven experiments), or total acids (seven experiments) in the experiments on the dogs. The shifts of the CO_2 capacity could not be correlated with those of the body fluids, for in seven experiments on dogs there was no consistent change of O_2 capacity or of the concentration of the serum as evidenced by serum proteins, total solids, or specific gravity.   Similarly, in the three cats the serum proteins remained unchanged (data not presented). The bicarbonate of the serum, like the CO_2 content of the whole blood, increased irrespective of the direction of the change of the CO_2 capacity of the whole blood.   The results of the experiment of Dog B are presented in Table I.

DISCUSSION

The present results yield an explanation of the discrepancies in the literature concerning the effect of the inhalation of CO_2 on the CO_2 capacity of the blood.   The variations are probably due to the length of time of exposure to the increased concentration of CO_2.   In a long series of experiments Shaw and Messer (1932) obtained results on dogs and cats anesthetized with sodium barbital similar to ours on unanesthetized subjects.

The fall in CO_2 capacity (Shaw and Messer) occurred in 5 minutes and was noted in dogs after they had respired a gas mixture containing 11 per cent of CO_2 through a tracheal cannula for 40 minutes.   In cats the same result was obtained after inhalation for 35 to 60 minutes.   Davies, Haldane, and Kennaway (1920–21), the only observers to use venous blood, noted a rise hardly exceeding the error of their method in the CO_2 capacity of the blood of humans.   It should be noted that this occurred after a mixture containing 6 per cent of CO_2 was inhaled in a gas chamber over a period of 2 hours.   Patients with emphysema, who had therefore endured high tensions of CO_2 for a long time, were found to have an

increased CO_2 capacity, both by Scott (1920) and Dautrebande (1925). Henderson and Haggard (1918) noted a rise of CO_2 capacity of dogs rebreathing through a mask a mixture starting approximately at 6 per cent of CO_2 and increasing to 10 and 20 per cent in 1 to $1\frac{1}{2}$ hours. This rise was observed even during the 1st hour and therefore resembled our results on Dog B.

Omitting consideration of these last results for the moment, we note that a diminution of CO_2 capacity occurred only in the observations made within the 1st hour of inhalation of CO_2, while a rise invariably resulted from a longer exposure to that gas. The fact that dogs did not invariably react with an early fall of carbon dioxide capacity may be due to a species difference. Shaw and Messer (1932) found a fall of only 2.1 volumes per cent in dogs, while in cats the decrease was 4.2 volumes per cent, yet both species breathed the same mixture (11 per cent CO_2). Our observations on human subjects exposed only to approximately 6 per cent CO_2 yielded an average fall of 3.01 volumes per cent.

Shaw and Messer (1932) have suggested that the decrease in CO_2 capacity is caused by a transfer of bicarbonate from the blood to the tissue fluids. It might also be due to the renal excretion of bicarbonate. The present results reveal no other cause for the fall in CO_2 capacity. It is possible that during the first half hour there might have been an accumulation of an organic acid which was then rapidly excreted in the urine. However, the concentration of lactic acid remained unchanged throughout the experiment. Ketone acids were not determined, but it is well known that organic acids accumulate in the blood in response to an alkalosis and not an acidosis. An analysis of the results reveals no evidence of a compensatory production of an acid, which would decrease the CO_2 capacity of the blood. Despite a small rise in CO_2 content of the serum the total acids showed no significant increase and the chlorides suffered no decrease, as might have been the case if a fixed acid were liberated.

Neither is the fall in CO_2 capacity due to a shift of fluids from the blood into the tissue spaces, for the concentration of the serum yielded no evidence for such changes.

The relations of the results of CO_2 content and CO_2 capacity are presented graphically with the aid of CO_2 dissociation curves (Fig. 1). The original position of the curve is OA and in some cases this

falls to *OB* during the first half hour in the CO₂ chamber. Later
the curves rise to *OC*. The CO₂ capacity of each at 40 mm. of
mercury tension of CO₂ is *1*, *2*, and *3* respectively. The original
CO₂ content is therefore *N* placed on *OA*. When tension is raised
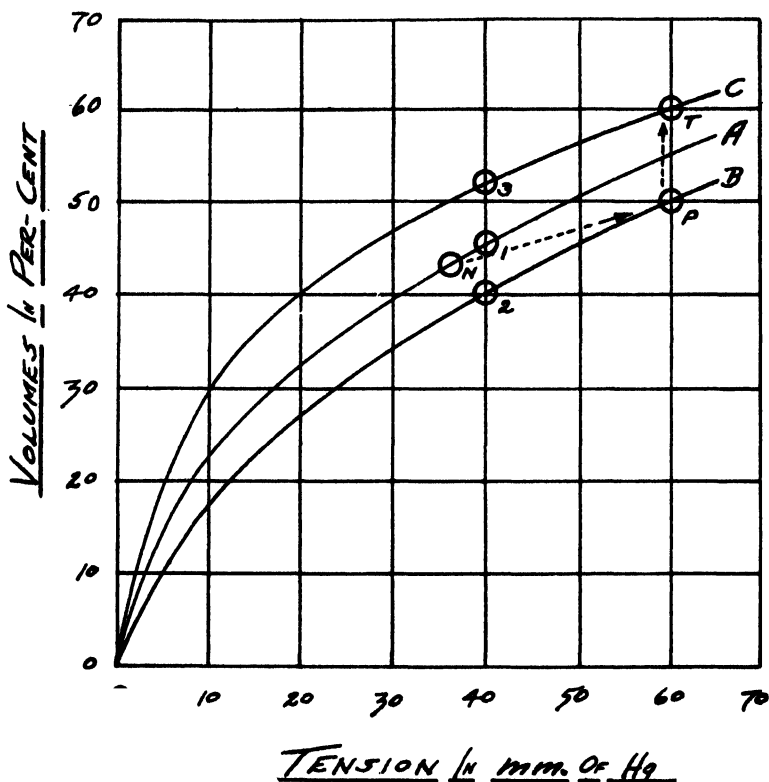
FIG. 1. Changes of CO₂ content and capacity during inhalation of CO₂,
showing diphasic variations of CO₂ dissociation curve. *OA*, original
position of CO₂ dissociation curve, *1* capacity, *N* content; *OB*, primary
fall of CO₂ dissociation curve, *2* capacity, *P* content; *OC*, secondary rise of
CO₂ dissociation curve, *3* capacity, *T* content.

in the CO₂ chamber, the content rises to a higher position, *P*,
which, nevertheless, is situated on the lower curve *OB*, as can be
established by determining the lower CO₂ capacity at 40 mm. (*2*).
Later when the capacity rises to *3*, the content assumes a new po-

sition, T, on OC determined by the CO_2 tension existing in the blood at that time.

SUMMARY

Seven observations were made on five human subjects, seven on four dogs, and three on each of three cats to test the effect of the inhalation of CO_2 (5 to 8 per cent) on the CO_2 capacity of the blood.

Periods of exposure to CO_2 of approximately half an hour produced a decrease of CO_2 capacity in all the human subjects and in three of the four dogs. Observations made at 55 minutes or later revealed a rise of CO_2 capacity in all instances. These results show that the compensatory increase of CO_2 capacity which serves to diminish the fall in pH is not the first response to inhalation of CO_2. A review reveals that most of the discrepancies of literature may be explained on this temporal basis.

A study of the acid-base equilibrium of serum yielded no evidence for the accumulation of an unknown acid to explain changes of CO_2 capacity. Similarly, an examination of the concentration of the serum revealed no shifts of fluid to account for the variations of the CO_2 capacity.

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LIVER INJURY BY CHLOROFORM, NITROGEN METABOLISM, AND CONSERVATION*

LIVER FUNCTION AND HEMOGLOBIN PRODUCTION IN ANEMIA

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We have observed in this laboratory that the healthy anemic dog will form much new hemoglobin during protein-fasting periods, particularly when iron is given during the fast. As much as 100 to 150 gm. of new hemoglobin may be produced as the result of iron feeding during a 2 week fast and obviously the hemoglobin must be derived from protein within the body. It was found that during this period of active hemoglobin production and fasting the nitrogen in the urine fell much below control levels and the conserved material could account for about one-half the new formed hemoglobin. The urea-ammonia urinary nitrogen was particularly concerned in the conservation and fell to very low levels.

One may inquire as to what organ may be related to this conservation reaction and suspicion would perhaps fall on the liver. It was decided therefore to study again the nitrogen metabolism of the dog subjected to severe liver injury (chloroform), both in the non-anemic and anemic states with intravenous iron administration during the latter. The liver appears to be one of the storehouses for nitrogenous intermediates. There is convincing evidence that the liver plays some rôle in the anabolism of body proteins (for example—fibrinogen). Following chloroform anesthesia, it would seem that the injured liver must play a very special part in its own repair, using for this purpose material stored in its least injured portions or in other storehouses of the body, or, as in the fasting anemic dog, extracting needed materials

from the normal catabolic stream. When the liver is severely injured by chloroform, we see little evidence for nitrogen conservation in anemia during periods of iron intake, but the expected formation of new hemoglobin goes on apace and the net total hemoglobin production is about the same with or without chloroform liver injury. This complex reaction is discussed below.

Studies of urinary nitrogen partition following chloroform anesthesia have been made by a number of investigators. There is general agreement that total urinary nitrogen increases. Failure of the body to deaminize amino acids, with consequent decrease in the percentage of blood and urinary urea-ammonia nitrogen and increase in the percentage of amino acid nitrogen, has been reported in the late stages of fatal chloroform poisoning. Marshall and Rowntree (8) found unusual amounts of amino acid nitrogen in the blood and urine of dogs immediately before death from chloroform poisoning. Stander (10) found abnormally small proportions of urea in the urine of patients just before death. Levene and Van Slyke (6), however, found no increase in the proportion of amino nitrogen in the urine of dogs severely injured by chloroform and phosphorus and they emphasize the extent to which liver injury must go before it affects amino acid metabolism. Increase in the excretion of creatine has been reported by Howland and Richards (5) and by Lindsay (7). The excretion of creatinine, on the other hand, has been shown to remain fairly constant by Howland and Richards (5), by Lindsay (7), and by Paton (9). Increases in blood uric acid were reported by Williamson and Mann (12).

Experimental Observations

All dogs used in these experiments were active, healthy adults. The anemic dogs were taken from the anemia colony in this laboratory and their histories are complete from birth. The care of these animals has been described elsewhere (11).

During the metabolism experiments the dogs were kept in galvanized iron metabolism cages in the laboratory, where they were under constant supervision. The urine was collected frequently as passed and was preserved by toluene and refrigeration. Each 2 day period was terminated by catheterization of the animal and rinsing of its bladder. Urine analyses were made on each 48 hour

specimen for the following constituents: total nitrogen by macro-Kjeldahl, ammonia by aeration, urea by the urease method with aeration, creatinine and creatine by the methods of Folin, and uric acid by the method of Morris and MacLeod. The dogs were caused to fast 2 days before urine collections were begun and 2 additional days before the administration of chloroform. By fasting we mean a discontinuance of food but a liberal supply of water. Narcosis was induced by ether and then chloroform was administered for 60 minutes by the drop method. Light surgical anesthesia was maintained. Symptoms of delayed chloroform poisoning developed within 48 hours, the intensity varying from experiment to experiment. No alarming symptoms were displayed, with the possible exception of Dog 33-14 (see experimental history, Table III). In four of the five experiments to be described, food was withheld from the animals for 2 weeks following the chloroform anesthesia. In the fifth experiment 40 to 50 gm. of glucose were given each day during the 2 weeks following the anesthesia, either by stomach tube or intravenously, depending on the animal's tendency to vomit.

Table I (Dog 34–5) presents data concerning a fasting period with chloroform anesthesia. This animal was about 2 years old and had been fed since weaning on a diet containing 50 per cent by weight of pig liver.

It will be noted that the rate of urinary nitrogen excretion more than doubled immediately following the anesthesia and remained above the control level throughout the experiment. The rate of excretion of urea and ammonia increased somewhat more rapidly than that of the total nitrogen and remained disproportionately high. The amount of creatinine in the urine increased very slightly following the administration of chloroform and then decreased somewhat, as one would expect in an uncomplicated fasting experiment. The amount of creatine increased sharply and remained high. The rate of excretion of uric acid reached a peak which was nearly 5 times as high as the control level. The figures for undetermined nitrogen are roughly proportional to those for total nitrogen throughout the experiment, but there is a slight tendency for the undetermined N percentage to fall as the urea-ammonia N percentage rises.

The highest levels of excretion of each of the urinary constitu-

ents determined were reached in Periods 2 and 3. It would seem that by far the largest portion of the necrotic liver cell material was being removed during this time.

TABLE I

Urinary Nitrogen Excretion As Influenced by Chloroform Anesthesia
Dog 34-5. Fasting experiment.

Period No.	Total N	Urea N + NH₃-N		Creatinine N		Creatine N		Uric acid N	Undetermined N
	mg.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	9,830	8,460	86.1	489	5.0	27	0.3	28	8.3

Chloroform anesthesia for 60 min.

2	23,160	20,180	87.1	515	2.2	326	1.4	96	8.9
3	25,170	22,620	89.9	468	1.8	602	2.4	131	5.4
4	17,210	15,060	87.5	400	2.3	336	2.0	67	7.8
5	15,010	13,080	87.1	379	2.5	309	2.1	43	8.0
6	12,570	11,070	88.1	344	2.7	237	1.9	36	7.0
7	11,630	10,520	90.4	338	2.9	155	1.3	27	5.2
8	11,560	10,450	90.4	305	2.6	197	1.7	25	· 5.1

The figures in the columns headed "mg." represent mg. of nitrogen excreted in successive 48 hour periods.

Experimental History during Metabolism Study Period (Table I)

Dog 34-5, bull mongrel male, adult. 18 days fasting. 60 minutes of chloroform anesthesia in forenoon of 5th fast day. Dog vomited during the night following the anesthesia and several times the next day. The urine sample (Period 2, Table I) was contaminated with vomitus and feces and the fourth, fifth, and sixth samples contaminated with feces. Dog was in good condition at end of experiment. Dog weighed 17.9 kilos on 5th day and 13.6 kilos on 19th day of experiment. Plasma fibrinogen 2nd day following anesthesia 84 mg. per cent, 3rd day 146 mg. per cent, and 4th day 206 mg. per cent. Icterus developed as usual. Bile pigment ++ in plasma on 2nd day following anesthesia, and + on 3rd and 4th days. Some bleeding from nostril.

The rate of excretion of nitrogen did not fall to a normal level in the latter part of the experiment, at which time repair and regeneration of liver tissue must have been taking place (4), and there is no diminution at this time in the urea-ammonia nitrogen percentage. This is in sharp contrast to the results of the metab-

olism experiments during regeneration of hemoglobin by anemic dogs (2).

The amount of surplus nitrogen appearing in the urine as a result of the chloroform anesthesia is far too great to have been derived from liver tissue alone.   If we accept 9.0 gm. (a figure which is probably too high) as the average amount of nitrogen which would have appeared in the urine every 2 days in a control experiment, then the additional nitrogen appearing in 2 weeks as a result of the administration of chloroform is 53.3 gm.   This is equivalent to 330 gm. of protein or more than 1500 gm. of fresh liver.   It is probable that this dog's liver weighed 400 to 550 gm.   It is evident that tissues other than liver must have been involved in the increase in nitrogen catabolism brought about directly or indirectly by the chloroform.

Table II offers comparison of the results of metabolism experiments on Dog 31-271 during fasting and with sugar ingestion. The sugar feeding experiment was done first and a 6 month period intervened between the first and second periods of chloroform anesthesia.

The experiment with zero food intake (Table II) gave very similar results to those shown in Table I.   The rate of excretion of urinary nitrogen was nearly doubled following the anesthesia and returned to the control level only at the end of the experiment. The percentage of urea and ammonia nitrogen was increased and remained high until near the end of the experimental period.   The amount of creatinine in the urine increased slightly and then decreased at nearly the normal rate for fasting dogs.   Creatine was excreted in fairly large amounts only in the 4 days following the anesthesia.   The rate of uric acid excretion rose to more than 6 times its initial value.   The ratio between the figures for undetermined nitrogen and those for total nitrogen remained nearly the same throughout the experiment.   The highest levels of excretion of the various urinary constituents were reached in the first 4 days following the administration of chloroform.

The sugar feeding experiment (Table II) shows a number of interesting contrasts with the fasting experiment on the same dog (No. 31-271).   The *conservation* of urinary nitrogen is very conspicuous and Period 8 shows a total N of 2.89 gm. in contrast to 4.57 gm. with fasting alone.   This illustrates the well established

TABLE II

Urinary Nitrogen Excretion Following Chloroform Anesthesia As Influenced by Fasting and by Ingestion of Sugar

Dog 31-271.

Period No.	Total N	Urea N + NH₃-N		Creatinine N		Creatine N		Uric acid N	Undetermined N

Fasting experiment

	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	4910	3800	77.5	368	7.5	0	0	17	14.7

Chloroform anesthesia for 60 min.

2	9270	7450	80.3	375	4.0	62	0.7	43	14.5
3	9090	7270	80.0	354	3.9	103	1.1	106	13.8
4	6480	5290	81.6	317	4.9	0	0	65	12.5
5	5620	4700	83.7	302	5.4	0	0	32	10.3
6	6330	5070	80.0	301	4.8	0	0	30	14 7
7	5110	4190	82.2	297	5.8	0	0	23	11.6
8	4570	3540	77.5	292	6.4	0	0	20	15.7

Sugar ingestion experiment

| 1 | 4990 | 4040 | 80.9 | 379 | 7.6 | 0 | 0 | 18 | 11.1 |

Chloroform anesthesia for 60 min.

2	7460*	6090	81.6	316	4.2	67	0.9	33	12.8
3	9000	7640	84.9	350	3.9	118	1.3	60	9.2
4	5170	4310	83.5	261	5.0	0	0	51	10.5
5	5210	4530	86.9	291	5.6	0	0	40	6.7
6	4140	3400	82.2	294	7 1	0	0	29	10.0
7	2840	2280	80.2	291	10.3	0	0	20	8.8
8	2890	2250	78.0	261	9.0	0	0	19	12.4

The figures in the columns headed 'mg." represent mg. of nitrogen excreted in successive 48 hour periods.

* Some loss of urine.

Experimental Histories during Metabolism Study Periods (Table II)

Dog 31-271, mongrel male, adult.

Fasting Experiment—18 days fasting. 60 minutes of chloroform anesthesia in forenoon of 5th day of fasting. Dog vomited during the night following the anesthesia and several times the next day. Urine sample in second period contaminated with vomitus. Dog in good condition at end of experiment. Dog weighed 14.4 kilos on 5th day, 12.6 kilos on 15th day,

and 12.2 kilos on 19th day of experiment. Plasma fibrinogen 2nd day following anesthesia 84 mg. per cent, and 3rd day 107 mg. per cent. Icterus present as usual. Bile pigment $+++$ in plasma on 2nd, 3rd, and 4th days following anesthesia.

Sugar Ingestion Experiment—5 days fasting. 60 minutes of chloroform anesthesia in afternoon of 5th fast day. 40 to 50 gm. of glucose given intravenously or by stomach tube daily thereafter, the total being approximately 600 gm. in 13 days. Dog vomited following sugar feeding by stomach tube on 4th, 5th, and 7th days following the anesthesia. No urine contamination. Dog in good condition at end of experiment. Dog weighed 13.2 kilos on 5th day and 11.85 kilos on 15th day of experiment. Plasma fibrinogen 2nd day following anesthesia 88 mg. per cent, and 3rd day 145 mg. per cent. Icterus developed as usual. Bile pigment $++$ in plasma on 2nd, 3rd, and 4th days following anesthesia.

protein-sparing action of carbohydrate by means of *conservation* of nitrogenous intermediates (4).

After liver injury with sugar feeding we know that active *liver cell regeneration* was going forward in Periods 4, 5, 6, and 7, and one may be surprised that there is no change in the urea-ammonia ratio, but we must not forget that we have a double reaction of great complexity. There is liver cell injury, with simultaneous autolysis of dead liver cells and formation of new liver cells. There is also some general body protein injury, presumably related to the breakdown and digestion of the dead liver cells. It is perhaps not surprising that the individual reactions of liver necrosis, liver repair, and body protein injury should give a confused picture, with the algebraic sum representing the urinary nitrogen partition. Some day we may be able to separate and give proper analysis to each of these several factors.

Table III gives the results of two metabolism experiments on anemic dogs. Each dog was subjected to chloroform anesthesia for 1 hour and was given colloidal ferric hydroxide by vein. Dog 33-14 was a young adult and was quite susceptible to chloroform poisoning. Dog 24-45, on the other hand, a 12 year-old hysterectomized animal with an anemia history of 11 years, was highly resistant. The experiment on this dog (No. 24-45) may serve as a good control for the successful experiment on Dog 33-14.

The results of the experiment on Dog 24-45 (Table III) show very little effect on the nitrogen metabolism of the chloroform anesthesia. No urinary constituent was increased in amount

TABLE III

Urinary Nitrogen Excretion by Anemic Dog As Influenced by Chloroform Anesthesia and Intravenous Iron Injections

Period No.	Total N	Urea N + NH₂-N		Creatinine N		Creatine N		Uric acid N	Undetermined N

Dog 33-14.   Anemia; fasting; iron by vein

	mg.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	5,120	3,930	76.8	278	5.4	94	1.8	16	15.7

Chloroform anesthesia for 60 min.

2	7,720	6,110	79.2	298	3.9	242	3.1	24	13.5
3	17,660	14,870	84.2	279	1.6	725	4.1	63	9.7
4	5,890	4,760	80.8	221	3.8	71	1.2	111	12.3
5	4,850	3,840	79.2	222	4.6	10	0.2	51	14.9
6	3,990	3,060	76.7	213	5.3	4	0.1	32	17.1
7	4,400	3,480	79.1	209	4.8	9	0.2	31	15.2
8	3,880	3,050	78.6	203	5.2	11	0.3	25	15.3

Dog 24-45.   Anemia; fasting; iron by vein; resistant to chloroform

| 1 | 10,670 | 9,040 | 84.7 | 483 | 4.5 | 239 | 2.2 | 30 | 8.3 |

Chloroform anesthesia for 60 min.

2	12,870	11,080	86.1	497	3.9	285	2.2	50	7.4
3	11,450	9,730	84.9	475	4.1	247	2.2	53	8.3
4	6,470	4,850	74.9	400	6.2	172	2.7	36	15.6
5	7,200	5,600	77.8	422	5.9	111	1.5	34	14.3
6	5,700	4,270	74.8	391	6.9	132	2.3	31	15.5
7	4,930	3,790	76.9	361	7.3	61	1.2	24	14.1
8	4,760	3,240	68.0	372	7.8	93	2.0	21	21.8

The figures in the columns headed "mg." represent mg. of nitrogen excreted in successive 48 hour periods.

Experimental Histories during Metabolism Study Periods (Table III)

Dog 33-14, coach mongrel female, adult, anemic.   18 days fasting.   60 minutes of chloroform anesthesia on afternoon of 5th day.   32 mg. of iron in form of colloidal ferric hydroxide given intravenously on each of 14 days, beginning with 1st fasting day.   Bleeding was difficult to stop after venous puncture on 2nd and 3rd days following chloroform anesthesia.   Normal blood (20 cc.) was given intramuscularly on 2nd day of intoxication.   Water by stomach tube was vomited.   Subcutaneous saline solutions were then given two or three times daily for 6 days.   Water by stomach tube was then

resumed. This animal had a very severe delayed chloroform poisoning and was very ill on the 2nd day following the anesthesia and for a few days thereafter. Dog was in good condition at end of experiment. Dog weighed 10.8 kilos on 5th day and 8.4 kilos on 19th day of experiment. Plasma fibrinogen before anesthesia 165 mg. per cent, 2nd day following chloroform 96 mg. per cent, and 4th day following 184 mg. per cent. On the 2nd day the plasma clotted with difficulty. Icterus was present as usual. Bile pigment $++$ in plasma on 2nd, 3rd, and 4th days.

Dog 24-45 (Table III), female bull mongrel, adult. 18 days fasting. 60 minutes of chloroform anesthesia on afternoon of 5th day. 32 mg. of iron in form of colloidal ferric hydroxide given intravenously on each of 14 days, beginning with 5th fasting day. 200 cc. of water daily by stomach tube. Dog in good condition at end of experiment. Dog weighed 23.4 kilos on 5th day and 21.1 kilos on 19th day of experiment. Plasma fibrinogen before anesthesia 249 mg. per cent, 2nd day following chloroform 226 mg. per cent, and 4th day following 198 mg. per cent. No icterus. Bile pigment in plasma negative.

comparable to the increases shown in Tables I and II. Periods 2 and 3 show a slight increase in total urinary N above the control period. We may assume that this reaction is due to a slight liver injury due to chloroform but that liver repair is prompt. This reaction is all over within 4 days (periods 2 and 3). The effect of the injection of iron, on the contrary, was marked. The rate of urinary nitrogen excretion fell from a level of 10.67 gm. per 48 hour period to 4.76 gm., while the urea and ammonia nitrogen percentage fell from 85 to 68. This is the typical picture which we have come to associate with conservation of material for hemoglobin construction (2, 3).

The results of the experiment on Dog 33-14 (Table III) offer a decided contrast. The effect of the chloroform anesthesia on N excretion was very great. The amount of nitrogen excreted on the 3rd and 4th days (Period 3) following the anesthesia was 3 times as much as that excreted in the 2 day control period. The urea and ammonia nitrogen percentage rose from 77 to 84. Creatine excretion increased almost 8-fold, rising to a point where almost 3 times as much creatine as creatinine appeared in the urine. About 7 times as much uric acid was excreted in Period 4 as during the control period. Taking the size of the dog into consideration, these results indicate a very severe destruction of liver and injury of other tissue. The clinical picture indicated a severe chloroform liver injury which might have resulted fatally but for injections of blood and saline solution.

A 12 kilo dog such as this should have a liver weighing perhaps 400 gm. Yet the excess urinary nitrogen during Period 3 alone (17.66 gm.−5.12 gm.) is equivalent to approximately 78 gm. of

TABLE IV

Iron by Vein during Fasting, with and without Chloroform Liver Injury

Dog 33-14.   Coach, female, adult.

Diet periods 1 wk. each Food, gm. per day	Food con-sump-tion	Weight	Plasma vol-ume	R.b.c.	Blood Hb level	Hb re-moved, bled
	per cent	kg.	cc.	millions	per cent	gm.
Bread 375, salm. 100, Kl. 20......	100	12 4	752		50	1.5
Chloroform 1 hr., Fe 32 mg., fast-ing..........................	0	10.0	474	7.1	76	23 8
Fe 32 mg., fasting..............	0	8 7	389	6.8	70	39.8
Bread 375, salm. 75, Kl. 20.......	100	9.8	600	5 7	59	43.0
" 375, " 75, " 20.......	100	10.3	578	4.4	55	25 8
" 375, " 75, " 20.......	100	10.8	645	4 0	50	21.3
" 375, " 75, " 20.......	100	11.2	669	4.1	45	1.3
" 375, " 75, " 20..... .	100	12 0	683	5.0	51	22 9
Basal output, 12 gm. Hb per wk. Total net Hb output, 119 gm. Total = 177.9						
Bread 375, salm. 75, Kl. 20.......	100	13.0	743	5.1	48	1.4
Fe 32 mg., fasting..............	0	11.7	585	6.3	74	26.6
" 32 " "	0	10.3	529	6.9	67	35.4
Bread 300, salm. 75, Kl. 20.......	100	9.2	498	5.7	67	22.5
" 375, " 75, " 20.......	100	10.5	581	5.7	62	35.1
" 375, " 75, " 20.......	100	11.1	660	5.5	53	34.7
" 375, " 75, " 20.......	100	11.8	674		54	22.2
" 375, " 75, " 20.......	100	12.0	648	4.7	52	12.3
Basal output, 12 gm. Hb per wk. Total net Hb output, 133 gm. Total = 188.8						

Salm. = salmon; Kl. = Klim.

protein or, had it all come from liver, to almost 400 gm. of liver tissue.

Table IV gives the necessary experimental data to show that the

anemic dog can make the expected amount of new hemoglobin when given iron by vein, whether the liver has been injured by chloroform or remains normal.   When fourteen doses of colloidal iron (32 mg. per day) are given by vein, the theoretical return of all this iron in new hemoglobin is 134 gm.   We see that this dog does actually return 133 gm. of new hemoglobin as the net amount above the control level when the liver is normal.   When the liver has been seriously injured, we observe a net hemoglobin production of 119 gm.   There is also a conspicuous shrinkage of blood volume at this time.   Although the amount of hemoglobin formed is less when the liver is injured, no stress can be placed upon this observation, as it falls within unexplained physiological variations. If the liver is concerned in this reaction, and we can scarcely believe that the liver is not an important factor in the metabolism of hemoglobin, then this function of the hepatic cells might be compared with the urea-forming activity of the liver.   It is well known that the liver is able to form urea, except in the last stages of extreme and usually fatal liver injury.

DISCUSSION

The words *internal metabolism* as related to proteins comprehend a vast unknown of complex reactions relating to proteins built in the body (*e.g.*, hemoglobin, plasma protein, organ proteins) from substances coming into the body usually by way of the intestinal tract.   Only occasionally do we glimpse a part of some reaction related to this great complex.   A case in point is the conservation of nitrogenous split-products to produce new hemoglobin during protein fasting periods in anemia.   This is a uniform reaction (2) which may be repeated again and again with considerable uniformity of pattern and is illustrated in Table III, Dog 24-45.

When to this reaction we add a serious liver injury what would be the resultant?   One might believe that more hemoglobin would be produced because more split-products are available (derived from the necrotic liver cells), or one might argue that because the liver is a very important organ related to the production of prehemoglobin materials, actually less hemoglobin would be produced. .   Practically the same amount of hemoglobin is produced with or without liver injury when the iron is given intra-

venously, and we may choose to believe that the two factors (excess split-products and impaired liver function) actually tend to neutralize each other.

When the liver is injured, there is a flood of nitrogenous material appearing in the urine (Tables I and III) which may well mask a moderate reaction of conservation aimed to produce more new hemoglobin in the presence of the stimulus of the associated anemia. As a matter of fact when we compare Dog 31-271 (Table II) and Dog 33-14 (Table III), we note that the anemic dog (Table III) actually does show less urinary nitrogen in the last four periods as compared with the non-anemic dog (Table II) and the urea-ammonia percentage is a little lower in the anemic dog. This may point to some conservation of nitrogenous material to form the needed hemoglobin.

When the liver is severely injured by chloroform, we know (4) that organ repair will go on even in the absence of any food intake, and with sugar intake the repair will be much more rapid and bring the liver back to a normal histological picture in about 10 days. Table II shows this type of reaction in the same dog and we note that with sugar feeding the loss of nitrogenous material in the urine is greatly reduced. We may well believe that from some of this conserved material the protein of the new liver cells is fabricated. Yet the ratio of urea and ammonia is unchanged during this period of conservation, which indicates a decided difference between new hemoglobin construction and new liver cell protein production. We note (Table II) also that there is a difference in the undetermined nitrogen, which is much reduced in the sugar-feeding experiment and may indicate some conservation of materials within this fraction aiming toward liver repair.

The *protein-sparing action of carbohydrates* during a protein fast may be at the source, due to energy supply in the form of sugar protecting the body proteins. The example given in Table II is one of the best proofs that sugar may *spare proteins* by an act of *conservation* which saves nitrogen to build protein from material which otherwise would contribute to the nitrogen of the urine. This has been discussed in detail in another paper (4).

When a *sterile abscess* is produced in a fasting dog (1), there is a large increase in urinary nitrogen and at the same time some fever, leucocytosis, and clinical intoxication. The tissue breakdown in

the abscess cavity is obviously responsible and all this response is due to disintegration of *host protein*. The destroyed protein will account for less than 10 per cent of the excess urinary nitrogen. Obviously the injured protein sets free toxic split-products which injure other body tissues and bring about the systemic intoxication as well as the excess urinary nitrogen. In like fashion the injured and *dead liver cells* (chloroform) can account for only a part of the excess urinary nitrogen and we must believe that toxic split-products escape from these injured liver cells. These poisons react on other body tissues to account for the rest of the excess urinary nitrogen. The fact that *creatine* may be conspicuously increased points to the muscles as one tissue probably injured in this general response—the muscles containing about 98 per cent of creatine within the body.

Chloroform anesthesia for 1 hour following a 4 day fast will usually cause a severe central necrosis of the liver lobules amounting to one-half to two-thirds of each liver lobule. In fact, some dogs will be fatally poisoned and die within 3 to 4 days and show four-fifths or more liver necrosis. The *fibrinogen* is produced in the liver and its concentration in the plasma falls when the liver is injured by a severe chloroform necrosis. With a severe liver necrosis at the end of 48 hours after the chloroform anesthesia the *blood fibrinogen* will be found to be 100 mg. per cent or below and this blood fibrinogen index is important in determining the presence of a severe liver injury (compare Dog 33-14, Table III, severe injury, and Dog 24-45, Table III, a trivial injury in an old dog). Icterus is always present with a severe liver injury of this type.

It is well known that the *urea*-forming capacity of the liver is lost only in the presence of an extremely severe or lethal liver injury. In contrast, the *uric acid* mechanism is disturbed in less severe liver injury and we note high uric acid figures in the dogs which show severe chloroform liver damage (Dog 33-14, Table III).

<div align="center">SUMMARY</div>

The fasting dog given iron by vein in the presence of anemia will form the expected amount of new hemoglobin, whether the liver is injured by chloroform anesthesia or is normal.

Only with severe or fatal liver injury does the dog lose its urea-

forming capacity, and we may argue likewise that the hemoglobin-building contributions of the liver are lost only in the final stages of liver injury and insufficiency. In these experiments reported above the liver can still form urea as usual, in spite of rather severe liver necrosis due to chloroform.

The uric acid excretion in these experiments is quite high and this is believed to be due in part to functional impairment of the liver.

There is a great increase in urinary nitrogen on the 4 days following chloroform anesthesia, and necrotic liver cells can account directly for only a part of the excess urinary nitrogen. It must be assumed that toxic split-products escape from the dead and autolyzing liver cells to cause general intoxication and tissue injury in the body elsewhere. The conspicuous increase in creatine urinary excretion suggests that muscle tissue is injured in this indirect fashion.

The fasting dog with anemia and chloroform liver injury can form new hemoglobin and regenerate new liver cells, while the body is eliminating the excess waste products due to the chloroform poisoning. This is a very complex admixture of injury, regeneration, and repair—an illustration of the flexibility of the internal metabolism of the body. It need excite no comment that at the present time we cannot attempt a satisfactory explanation of the algebraic sum of these reactions as reflected in the total urinary nitrogen.

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THE METABOLISM OF *d*-XYLOSE*

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The advocacy of the use of xylose as a measure of renal function makes it desirable to know more about the fate of this sugar in the animal organism. When xylose is taken by a normal man, about one-third is excreted in the urine in 8 hours, at which time excretion is apparently ended. Undoubtedly, a variable amount of the sugar is excreted by the bowel as diarrhea is common after taking it. The question naturally arises, is any of the xylose utilized? Miller and Lewis (1) have investigated this subject and could find no evidence of glycogen formation in the white rat. They observed an increase in the pentose content of blood, liver, and kidney, but no change in the pentose content of muscle. An increase in blood glucose occurred, but the authors rather questioned its significance. This change in blood glucose, resulting from the administration of xylose to rabbits, had been observed previously by Blanco (2). The following experiments were undertaken in order to obtain further information about the metabolism of this sugar.

Methods

The white rats used were of a uniform strain and were fed the same diet. The composition of this diet was as follows: whole wheat 55, dried milk (Klim) 25, dried beef muscle 12, dried yeast (Harris) 5, sodium chloride 2, and calcium carbonate 1 gm. The experimental animals fasted 24 hours and were then given 1.5 cc. of 50 per cent xylose solution by stomach tube, according to the tech-

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nique of Cori (3).   After an absorption period of 3 hours, sodium
amytal was given intraperitoneally.   Tissue samples were then
removed and their carbohydrate content determined by the pro-
cedures recently described (4).   Muscles and liver were frozen *in
situ,* but the kidneys were removed and dropped into liquid air
after the liver had been frozen.   Blood samples were obtained
from the heart.   The other tissues were taken from animals which
had not been bled, since bleeding may affect their content of glyco-
gen and lactic acid.   In determining the amount of xylose ab-
sorbed, the gastrointestinal tract was slit open and washed with
hot water, after which it was cut into small pieces and transferred
to a 250 cc. volumetric flask.   The flask was then half filled with
boiling water and shaken.   After cooling, the mixture was made
up to volume and 25 cc. were treated with 5 cc. of N H_2SO_4, 5 cc. of
10 per cent $ZnSO_4$ solution, and 7.8 cc. of N NaOH.   The volume
was then made to 55 cc. and the solution filtered.   The usual treat-
ment with Lloyd's reagent and permutit was omitted.   1 cc. of
the filtrate was a suitable amount to use in the case of the rats
given xylose.   The control animals were fasted 24 hours and then
killed 3 hours later.

Considerable time was spent in trying to determine the pentose
content of tissues by the McCance (5) method, as modified by
Miller and Lewis ((1) p. 141), but the results obtained were not
sufficiently accurate to justify its use.   The estimation of the non-
fermentable reducing substances appears to give much more reliable
values.

EXPERIMENTAL

The results are presented in Table I in which the maximal,
minimal, and mean values are recorded.   The mean deviation of
the mean has been calculated with the formula

$$\cdot = \sqrt{\frac{\Sigma d^2}{(N-1)\,N}}$$

the significance of which has been discussed by Scott (6).

Glycogen—No significant changes in the glycogen content of
the liver or muscles occurred as the result of administering xylose.
The sex difference in the glycogen content of these tissues was
clearly apparent.

Table I

Carbohydrate Content of Tissues of Control and Experimental Rats

The results are expressed in mg. per 100 gm. except for blood, in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose. Figures in bold-faced type represent values for the rats given xylose.

Substance determined	Maximal	Minimal	Mean	Mean deviation of mean	No. of rats Males	Females
Glycogen, liver............	1847	106	555	127	15	
" "	**1583**	**58**	**624**	**141**	**13**	
" "	603	41	215	46		14
" "	**482**	**9**	**152**	**44**		**12**
" muscle.........	749	546	661	15.7	14	
" "	**822**	**597**	**684**	**20.5**	**14**	
" "	720	505	611	21.2		13
" "	**700**	**457**	**571**	**21.7**		**14**
Lactic acid, liver.........	29 7	10.7	19.5	1.4	10	5
" " "	**33.8**	**7.8**	**17.6**	**1.8**	**4**	**8**
" " muscle.......	30.5	13.1	21.6	1.4	9	5
" " "	**31.9**	**10.8**	**20.4**	**1.8**	**7**	**8**
" " blood........	21.8	11.9	15.5	1.1	4	7
" " "	**19.5**	**9.5**	**13.8**	**0.9**	**4**	**8**
Fermentable reducing substances						
Liver	219.5	81.9	127.5	8.9	9	5
"	**194.9**	**90.7**	**145.7**	**8.8**	**7**	**6**
Muscle...............	32.2	9.1	19 6	1.6	10	6
"	**33.8**	**5.3**	**16.8**	**2.5**	**8**	**6**
Kidney..........	68.4	36.7	56.5	2.5	9	6
"	**128.3**	**10.8**	**74.0**	**7.9**	**8**	**6**
Blood.................	91.5	59.0	73.8	2.6	6	9
"	**95.9**	**72.2**	**85.5**	**1.4**	**5**	**10**
Non-fermentable reducing substances						
Liver...................	47.3	0.0	11.7	3.0	9	5
"	**119.6**	**45.6**	**68.0**	**5.3**	**7**	**6**
Muscle...............	31.0	4.3	12.0	2.1	10	6
"	**36.3**	**6.5**	**22.7**	**2.8**	**8**	**6**
Kidney...............	13.4	4.9	7.6	0.6	9	6
"	**202.4**	**34.7**	**106.1**	**15.1**	**8**	**6**
Blood.................	6.7	3.0	4.9	0.3	6	9
"	**46.8**	**19.6**	**27.0**	**2.1**	**5**	**10**

Lactic Acid—The administration of xylose did not change the amounts of lactic acid in the liver, muscles, and blood.

Fermentable Reducing Substances—These substances were increased in the liver, kidney, and blood but there was a slight decrease in the muscles as a result of giving xylose. None of these changes was significant except the increase in blood glucose. The blood glucose of the control rats was 73.8 ± 2.6 and that of the rats given xylose was 85.5 ± 1.4 mg. per 100 cc.

Non-Fermentable Reducing Substances—The reducing substances not fermented by yeast increased in all of the tissues. These changes were all significant. The observed increases follow: liver, 11.7 ± 3.0 to 68.0 ± 5.3; muscle, 12.0 ± 2.1 to 22.7 ± 2.8; kidney, 7.6 ± 0.6 to 106.1 ± 15.1 mg. per 100 gm.; and blood, 4.9 ± 0.3 to 27.0 ± 2.1 mg. per 100 cc. The greater variability in the pentose content of the kidneys of the animals given xylose may be accounted for by the different amounts of xylose in the urine included in the samples.

Absorption Coefficient of Xylose—The amount of xylose absorbed from the gastrointestinal tract varied from 49.0 to 106.6 mg. per 100 gm. of rat per hour, with an average of 73.5 mg. for fifteen rats. This value is considerably greater than that of 46 mg. found by Miller and Lewis ((1) p. 133), and of 28 mg. reported by Cori (3).

<center>DISCUSSION</center>

The values for the non-fermentable reducing substances, 3 hours after the administration of xylose, show that the sugar had passed into the blood, liver, muscles, and kidneys. These results are in agreement with those of Miller and Lewis ((1) p. 141) except that they found no change in the pentose content of muscle after xylose was given. It is perhaps well to point out that the pentose content of liver, kidney, and muscle appears to be much greater when determined by the furfural method. Thus, Miller and Lewis obtained the following values for their control rats: liver, 53 mg.; kidney, 50 mg.; and muscle, 91 mg. per 100 gm. Our corresponding values were 11.7, 7.6, and 12.0 mg. per 100 gm. The figures for the pentose content of the blood of the control and experimental animals agreed well by both methods.

The only significant effect of xylose administration upon the

normal body carbohydrates was the resultant increase in blood glucose. This finding agrees with that of Blanco (2) and of Miller and Lewis ((1) p. 141). We think there can be no doubt that this is a real increase. The liver and kidneys also showed increases in fermentable reducing substances, and these were decreased in the muscles. These changes were small but they might be interpreted as indicating tendencies toward mobilization of glucose. Such an effect might result from the replacement of a normal carbohydrate by a foreign sugar. For a time we thought that this change might be an expression of a toxic effect of xylose upon the tissues. It was observed that the livers of rats given xylose show what is commonly called albuminous degeneration, that is, the liver cells are full of pinkish-staining granules, and they appear swollen. These changes led us to inquire further into the possible toxic effects of xylose. To this end twelve young rats, just after weaning, were fed our control diet to each 100 gm. of which 5 gm. of xylose had been added. Diarrhea lasting 3 or 4 days was produced in all of the animals. The growth of these rats was normal and they were in good condition. They were killed after they had eaten the diet for periods varying from 47 to 64 days. Four were killed after a 27 hour fast and their tissues analyzed. The results were comparable with those of our control animals, showing that whatever xylose accumulates in the tissues disappears rather quickly. The livers of two non-fasted rats showed a more marked granular condition of the cells than did those of the fasted animals. It appears that, although the administration of xylose produces abnormal changes in the liver, no serious permanent effects ensue. The kidneys of these rats were normal.

It remains to be considered whether the observed increase in blood glucose may be due merely to withdrawal of water from the blood. This seems possible since rats which have been given xylose are extremely thirsty and the gastrointestinal tract is filled with fluid 3 hours after its administration. We have determined the moisture content of liver, muscle, kidney, and blood without finding evidence of changes in the first three due to the administration of xylose. In the case of blood, concentration occurred. The water content of the blood of three control rats varied from

79.7 to 80.5, with an average of 80.3 per cent. This corresponds with the generally accepted figure. The values for five rats given xylose varied from 75.7 to 79.1, with an average of 77.6 per cent. The water content of the blood was, therefore, reduced to the extent of 3.5 per cent. A calculation shows that the observed increase in blood glucose is still significant after correction for the change in water content.

Marble and Strieck (7) investigated the respiratory metabolism after the ingestion of xylose. They observed a small rise in the respiratory quotient of normal and phlorhizinized dogs and in normal and diabetic men. Determination of the plasma carbon dioxide capacity showed no evidence of acidosis, which would increase the output of carbon dioxide. The authors concluded that oxidation of xylose had taken place. These experiments and those designed to show changes in the body carbohydrates indicate that xylose is, at the most, of little availability to the animal organism.

SUMMARY

The administration of xylose to white rats increased the content of non-fermentable reducing substances in the liver, muscles, kidneys, and blood. No changes in the glycogen content of liver and muscles nor in the lactic acid content of liver, muscles, and blood were observed. The blood glucose was significantly increased as a result of giving xylose.

The histological examinations of the tissues were made by Dr. F. M. Medlar. We are indebted to the United States Bureau of Standards for generous supplies of pure xylose.

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SELF-SEALING VESSELS FOR STORAGE OF SOLUTIONS USED IN THE VAN SLYKE GASOMETRIC METHODS

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The two types of vessels described below were designed (a) to eliminate the glass stop-cock in the alkaline stream of the vessel used by Van Slyke, Page, and Kirk (1) and the modified Hempel pipette of Van Slyke ((2) p. 109) and (b) at the same time automatically to provide a mercury seal for the tip of the delivery tube of the Guest-Holmes (3) vessel and of the simple soda-lime tube of Van Slyke ((2) p. 363). They are especially convenient for those methods in which the solution is passed through a mercury seal in the cup of the Van Slyke pipette and its volume measured in the chamber, for example, for the storing and transferring of 0.5 N NaOH in the carbon combustion method of Van Slyke, Page, and Kirk.

Both types of storage vessel are used in the same manner. With the desired amount of mercury in the cup of the Van Slyke pipette, the vessel is held so that the tip of the delivery tube barely dips into the mercury. With the thumb placed over the small hole in the rubber bulb, the bulb is squeezed until any air or solution below the mercury seal in the delivery tube has been expelled and the drop of mercury in the tube is merged with the mercury in the cup. With the tip still under mercury, the bulb is then released, and the rubber gasket on the tip of the delivery tube is fitted snugly into the bottom of the cup. With the upper cock of the Van Slyke pipette open and the leveling bulb on the lower ring, the solution is drawn into the chamber by manipulation of the lower cock. During this transfer of solution, the mercury in the storage vessel is displaced in such a way that a slight negative pressure is produced at the tip of the delivery tube. As the tip is lifted under the mercury in the cup, a drop of mercury is drawn into the end of the delivery tube, thus resealing it.

It is desirable to have a little water above the mercury in the cup of the Van Slyke pipette. This makes it easier to be certain that air is not trapped in or on the tip. It also facilitates washing the lower end of the bore of the delivery tube when this is considered desirable. To do this, water is drawn in and out by moving the drop of mercury up and down in the delivery tube, and is then ejected ahead of the mercury until the mercury is merged with that in the cup. After the transfer of solution, water is automatically drawn up into the tip below the drop of mercury, as the delivery tube is withdrawn, and serves as a wash preceding the next transfer of solution.

The type shown in Fig. 1, A is the lighter and more easily made of the two forms, but protects only against CO_2. That protection is adequate against CO_2 is shown by the fact that a strongly alkaline phosphate solution has been stored for nearly 2 years without producing any change in the blank in the method in which it is used.

The body of the vessel is a 100 cc. Kjeldahl flask. The construction of the trap, and its attachment to the flask and to the delivery tube, are shown sufficiently by the drawing. A trap 50 mm. long made of 16 mm. tubing is satisfactory. The dimensions of the various parts are not critical, except that with a delivery tube of approximately 1.5 mm. bore 2 mm. was found to be the most satisfactory size for the inside diameter of the inner tube of the trap.

The soda-lime cartridge is a glass tube as large as will slip into the neck of the Kjeldahl flask, constricted at one end or fused to a small tube which will pass through a 1-hole rubber stopper and fit snugly in the opening of the rubber bulb. (This makes it possible to renew the soda-lime without emptying out the alkaline solution.)

Enough mercury is run in through the mouth of the flask to fill the trap to a depth of about 30 mm. A little more or less does no harm. The soda-lime tube is put in place. The vessel is then filled from the chamber of the Van Slyke pipette by elevating the leveling bulb and opening the stop-cocks while the rubber tip on the delivery tube is pressed against the bottom of the cup. After the vessel is filled, the delivery tip is sealed in the usual manner.

A simpler trap, consisting of a U-tube and bulb formed in the

lower end of the delivery tube, is shown in Fig. 1, *B*. Its only advantage is ease of construction. One such trap may operate with entire satisfaction, while another apparently identical one may persistently fail to lift a satisfactory mercury seal out of the cup of the Van Slyke pipette. A longer bulb tends to make the operation more reliable, but this is still not as satisfactory as the trap shown in Fig. 1, *A*.
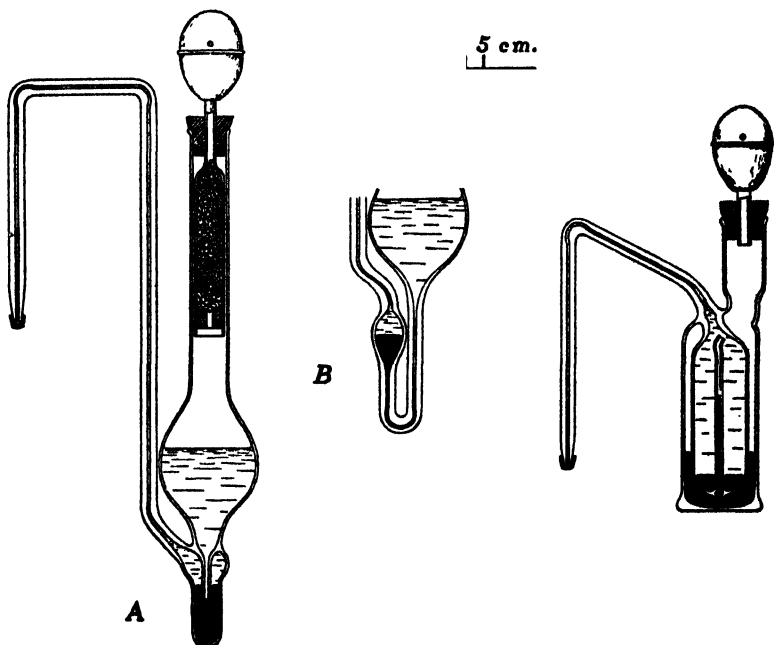
FIG. 1. Self-sealing vessels for storage of solutions used in the Van Slyke gasometric methods. *A* and *C* are the two types of vessels described in the text, *B* a simpler but less satisfactory alternative form of *A*.

A second type, Fig. 1, *C*, has the double advantage that it separates both surfaces of the stored solution by mercury from contact with the air, and that it stands on its own base, requiring no stand or support as do all of the other vessels mentioned.[1]

[1] A vessel which stands on its own base was informally exhibited by Dr. A. Baird Hastings at the meeting of the Federation of American Societies for Experimental Biology, at Detroit, April, 1935.

In the second type of vessel, the inner storage bulb is made of 32 ' mm. tubing and has an outside length of 80 to 85 mm.   The outer cylinder is made of 41 mm. tubing and has an outside length of 105 to 119 mm.   The tube between the storage bulb and the outer cylinder at the top is melted to a small bore with thick walls for greater strength and to reduce the volume of mercury which cannot be displaced by the solution in filling.   (The thickened periphery at the bottom of the vessel (optional) is intended only to decrease the amount of mercury required which does not take part in the operation of the apparatus.)

The delivery tube is made of capillary tubing having a bore of not less than 1 mm. and not more than 1.5 mm.   (The lower limit is to avoid too great movement of the seal with changes in the temperature of the laboratory.)   The vertical part of the delivery tube should be far enough from the body of the vessel so that it does not interfere with convenient use by bringing the vessel too close to the stop-cock of the Van Slyke pipette.   This portion of the delivery tube is made at least 12 cm. long, so that there will be no danger of the mercury seal's being drawn back into the bulb.   It is desirable that the bore of the delivery tube be not constricted in drawing down the tip, since a tapering bore results in the seal's being drawn up farther into the tube.

The innermost tube, in the storage bulb, has a bore approximately the same as or slightly larger than that of the delivery tube. If it is too small, too short a mercury seal will be drawn into the delivery tube.   If it is too large, the seal in the delivery tube will be drawn up too high.

The dimensions of the cup on the top of the vessel are limited only by convenience.   The opening into the vessel should be large enough to let mercury through freely, without putting pressure on the contents of the vessel.

If one wishes to avoid the somewhat difficult glass blowing involved, a vessel has been found to be satisfactory in which only the storage bulb, delivery tube, and the tube inside the bulb are blown in one piece.   The whole is then assembled by passing the delivery tube and the stem of the cup through a 2-hole rubber stopper, and forcing the stopper into the open end of a 250 cc. cylinder which has been cut off at a suitable length.

To fill the second type of vessel (Fig. 1, C), a heavy walled

rubber tube of small bore, connected with a glass tube with a rubber tip, is attached to the delivery tube of the vessel. The vessel is then filled with mercury through the cup until the mercury appears at the tip of the connecting tube, and the connecting tube is closed with a screw clamp. The vessel is then inverted over a large beaker, and supported in this position on a ring stand. The glass tip of the connecting tube is placed firmly against the bottom of the cup of the Van Slyke pipette, and solution from the chamber is forced over into the storage vessel. The mercury displaced by the solution falls into the beaker.

As soon as the storage vessel has been filled, the connecting tube is again clamped, the vessel is reverted, and mercury is added through the cup to the proper level, after which the connecting tube is detached. The rubber tip of the delivery tube and the rubber stopper carrying the rubber bulb are replaced, and a mercury seal is drawn into the delivery tube in the usual manner. With careful manipulation of the rubber connection between the rubber and glass tubes, bubbles entering the solution from the connecting tube can be avoided.

If the extraction has to be carried out in two portions, a little mercury from the chamber of the Van Slyke pipette may be forced into the connecting tube, which is then clamped, to serve as a seal while the next batch of solution is being extracted.

Mercury is added through the cup of the vessel from time to time as it is required to take the place of the reagent taken out.

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THE HYDROLYSIS OF STARCH BY HYDROGEN PEROXIDE AND FERROUS SULFATE*

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In 1924 Mathews (1) expressed the view that all matter exists in two forms, stable and reactive, due to the energy content of the molecule. On this basis he postulated that enzymes are substances which by their presence facilitate the transfer of energy from some external source to the substrate, thus raising its energy content and making it labile. The work of Hill (2), Boyd (3), and Sigal (4) from this laboratory has added evidence to support this view.

Since oxygen is such a common source of energy for living matter, it is quite possible that amylase is a part of an oxidation-reduction system, or is activated by such a system. On this hypothesis was based a series of experiments to ascertain just how closely the action of an oxidation-reduction chain upon starch parallels the action of amylase. Hydrogen peroxide and ferrous sulfate (Fenton's reagent) were chosen as a suitable oxidation-reduction system for the basis of experimental study.

Gatin-Gruzewska (5) and Gerber (6), using hydrogen peroxide alone, and Durieux (7), using hydrogen peroxide and ferric chloride, had shown that starch is broken down by means of these substances with the formation of dextrins and reducing substances. All three concluded that the action was analogous to that produced by diastase. Omori (8), using several heavy metals with hydrogen peroxide upon starch, secured the hydrolysis, but concluded that the action was quite different from that of enzymes.

The experiments were carried out according to the following scheme: 10 cc. of a 1 per cent soluble starch (Kahlbaum) solution, 2 cc. of acetate buffer (pH 3.8), 10 cc. of 1 per cent hydrogen peroxide (commercial 30 per cent diluted to 1 per cent), and 2 cc. of 0.01 M ferrous sulfate in a large test-tube were covered with toluene and placed in a bath at 37°. At various intervals small portions were removed and tested with dilute iodine and the optical rotation was observed. At other intervals small aliquots were taken and the acidity and cupric reducing power by the Bertrand method were noted.

As the reaction proceeded, the solution became water-clear and greatly reduced in viscosity. There was considerable production of cupric reducing substances (Table I) and a continuous decrease in the optical rotation of the solution (Table II). The tube containing 2 cc. of ferrous sulfate showed a decrease in optical rotation of 15.3 per cent of the original rotation in 3 hours. The tube containing 4 cc. of ferrous sulfate showed a decrease of 84.0 per cent in the same time. 6 cc. caused a decrease of 84.8 per cent in the 2½ hours, while 8 cc. effected a decrease of 82.7 per cent in 2 hours.

The color produced by addition of dilute iodine changed from blue to red and finally to colorless as the reaction proceeded. The rate of splitting of starch was directly proportional to the amount of iron added (Table II).

In addition to ferrous sulfate, which has been shown to be very active in the catalysis of the action of hydrogen peroxide on starch, several other salts, which show some catalytic action, were tested. Ferric salts are of the same order of efficiency as the ferrous. Copper and manganese salts are greatly inferior to the iron. Complex salts, ferro- and ferricyanides, have very little effect, while manganese dioxide has no effect whatever. The efficiency of these salts bears no relationship to their peroxidase activity as measured by the benzidine reaction. Copper is by far the most efficient as a peroxidase, ferrous and ferric iron being considerably weaker. Manganese, which is as effective in the catalysis of starch as copper, has practically no peroxidase action.

Tests were made upon the solution to ascertain if the action were a true hydrolysis with the liberation of free glucose or maltose.

After the achromic point with iodine had been reached, the solution reduced alkaline copper sulfate in the cold.   This indicated a much stronger reducing agent than the simple sugars.   The addition of

TABLE I

Production of Reducing Substances

Reaction mixture: 10 cc. of 1 per cent starch, 2 cc. of acetate buffer (pH 3.8), 10 cc. of 1 per cent H_2O_2, 5 cc. of 0.01 M $FeSO_4$.   Temperature 37°.

	Time	0.05 N $KMnO_4$	Cu	Iodine reaction
	hrs.	*cc.*	*mg.*	
Control, no H_2O_2, no $FeSO_4$......	24	0.60	1.89	Blue
" H_2O_2, no $FeSO_4$.........	24	6.65	20.94	Deep violet
Reaction mixture................	24	54.30	171.05	Colorless
" "	20	51.00	160.70	"
" "	18	52.90	166.60	"

TABLE II

Change in Optical Rotation during Reaction

Reaction mixture: 10 cc. of 1 per cent starch, 2 cc. of acetate buffer (pH 3.8), 10 cc. of 1 per cent hydrogen peroxide, 0.01 M $FeSO_4$ added as indicated.   Temperature 37°.   Water added to make total volume of 30 cc.

Experiment No.	0.01 M $FeSO_4$	Iodine reaction		Angle of rotation (beginning)	Angle of rotation				Reduction	Fuchsin test
	cc.	*hrs.*		*degrees*	*hrs.*	*degrees*	*hrs.*	*degrees*		
1 (No H_2O_2)	2	20	Blue	1.50	3	1.50	20	1.49	−	−
2	0	28 *min.*	Red	1.45	20	1.40	28	1.27	+	−
3	2	30	Colorless	1.50	3 *min.*	1.27	20	0.30	+	+
4	4	15	Pink	1.45	15	1.14	2.5	0.24	+	+
5	6	15	"	1.45	15	0.99	2.5	0.23	+	+
6	8	20	Colorless	1.45	15	0.91	2	0.26	+	+

phenylhydrazine caused the formation of an orange amorphous precipitate which turned brown on standing but could not be induced to crystallize. Glucose or maltose as such were not present. The solution was tested with fuchsin-sulfur dioxide

reagent and with sodium bisulfite for the presence of free aldehyde. Both of these tests were strongly positive, indicating that the simple sugars were further attacked with the liberation of a free aldehyde group. Neither hydrogen peroxide nor iron interferes with any of these tests. There was no change in these tests after the removal of hydrogen peroxide by the use of manganese dioxide. Added amounts of hydrogen peroxide or ferrous sulfate to these tests, carried out upon known solutions, had no effect.

Concentration of the reaction mixture *in vacuo* at 30–40° gave a distinctly acid distillate which did not reduce alkaline copper and did not restore the color to fuchsin-sulfur dioxide, but gave a strong reduction of ammoniacal silver nitrate. This was concluded to be probably formic acid. There was considerable acidity left in the residual solution. The acidity and aldehyde tests are considerably increased by the addition of glucose or maltose to the reaction mixture.

A solution of glucose or maltose, when treated with hydrogen peroxide and ferrous sulfate, increased in acidity and decreased in optical rotation. Formic acid and free aldehydes are produced. Phenylhydrazine does not give the characteristic osazone, but an amorphous precipitate which could not be induced to crystallize. These tests are identical with those given by the solution of starch after treatment with hydrogen peroxide and ferrous sulfate, and upon this basis the conclusion is reached that glucose, and possibly maltose, is formed in the course of the reaction only to be further hydrolyzed and oxidized to form acids and free aldehyde.

By the use of ethyl alcohol, there may be precipitated from the reaction mixture substances which have the properties of the dextrins formed by the action of acids or enzymes upon starch (Table III). By precipitating these substances while the solution still produced a red color with iodine (i), there was obtained a gummy residue which was quite soluble in water. Dialysis of this solution for 10 days removed all cupric reducing power, but left a solution which gave a deep red color with iodine and showed considerable optical activity.

$$[\alpha]_{5461}^{24} = \frac{100 \times 2.23}{1.0 \times 1.15} = +193.8°$$

Hydrolysis of this substance gave a quantitative yield of glucose.

TABLE III

Precipitation and Dialysis of Dextrins Precipitated from Reaction

Soluble starch

(Treated with H_2O_2 and $FeSO_4$ until red color is produced with I_2)

$| C_2H_5OH + NaCl$

(i) Residue   (i, A) Filtrate
- Clear gummy mass
- Red color with I_2
- Strong reduction (Benedict's)
- Dextrorotatory

Clear watery solution

$| C_2H_5OH + NaCl$

(ii) Residue   (ii, A) Filtrate
(Same as above, rotation   (Combined with i, A,
and reduction less)   alcohol removed)
- Reduction
- Formic acid
- Free aldehyde
- Acids
- No osazone

Dialyzed in cellophane

(Against distilled water)   *(Against running tap water 10 days)*

(iii) Residual solution   (v) Dialysate   (iv) Residual solution
- Rotation less   - Reduction   - No reduction
- Reduction less   - Dextrorotatory   - Less rotation
- Red color with I_2 - No osazone   - Molisch +
- No osazone   - No osazone

HCl (boiled)   HCl (boiled)   HCl (boiled)

Sugar solution   Sugar solution   Sugar solution
- More reduction   - More reduction   - Reduction
- Less rotation   - Less rotation   - Less rotation
- Glucosazone formed   - Glucosazone formed   - Glucosazone formed

The concentrated diffusate (ii, Table III) from the dialysis of the erythrodextrin was found to possess considerable optical rotation and some reducing power.

$$[\alpha]_{5461}^{24} = \frac{100 \times 1.0}{1.0 \times 0.794} = +138.5°$$

$$[\alpha]_{5490}^{24} = \frac{100 \times 0.963}{1.0 \times 0.794} = +122.4°$$

R (cupric reducing power) = 23.8 (maltose = 100)

This is in fair agreement with the constants for amylotriose from bacterial degradation of starch, given by Pringsheim (9).

$$[\alpha]_{5890}^{20} = +124.5°, \; R = 22.5 \text{ (maltose = 100)}$$

Hydrolysis of this solution by dilute acid increased the reducing power and decreased the optical rotation to that of a solution of glucose, and from the hydrolyzed solution the characteristic crystals of phenylglucosazone were obtained.

Precipitation of the original mixture, after the achromic point had been reached, yielded a gummy precipitate (i, Table III) as in the case of the erythrodextrin. This substance was soluble in water but gave no color with iodine. Prolonged dialysis removed all reducing power, but the solution still showed optical activity.

$$[\alpha]_{5461}^{24} = \frac{100 \times 0.45}{1.0 \times 0.25} = +180.0°$$

Hydrolysis of this solution by acid caused a reduction of optical rotation and an increase in the cupric reducing power until these agreed with those of a solution of glucose. The diffusate (v) from the dialysis of the achroodextrin was found to possess the following constants.

$$[\alpha]_{5461}^{24} = \frac{100 \times 0.81}{1 \times 1.58} = +52.53°$$

R = 4.1 (maltose = 100)

Hydrolysis of this solution increased the reducing power and decreased the optical rotation to some extent. This solution was

undoubtedly a mixture of the oxidation products of glucose containing a small amount of a substance of high optical rotation.

Hydrogen peroxide and ferrous sulfate react with starch to produce a hydrolysis. The opalescence of the starch is lost, its viscosity and optical rotation are reduced, its ability to reduce alkaline copper is increased, and from the reaction there may be isolated dextrins and the oxidation products of the simple sugars. With the exception of the further oxidation of the simple sugars produced, these characteristics are identical with those produced by enzymic or acid hydrolysis of starch. On this fact is based the conclusion that the action of the system is a true hydrolysis, analogous to that produced by the amylolytic enzymes.

The rôle of the metal in the reaction was adduced from several facts. First, the presence of iron or similar metal appears to be necessary for the hydrolysis. Although metal-free starch was not used in the experiments, the rate of the reaction without added metal was very slow, requiring several days to go to completion. The dependence of the rate of hydrolysis upon the amount of added iron (Table II) indicates that the complete removal of iron would cause the hydrolysis to be immeasurably slow.

Iron has the ability to catalyze the decomposition of hydrogen peroxide as does heat and alkalinity. The fact that the ability of the metals to catalyze the hydrolysis of starch is not in the order of their ability to catalyze the decomposition of the peroxide indicates that the iron plays a part other than the mere liberation of energy from the peroxide. The decomposition of the peroxide by heat or alkalinity does not cause hydrolysis of the starch in a measure to be expected from the amount of energy liberated.

The starch, peroxide, and iron must be in the same solution for the hydrolysis to occur, indicating a loose chemical union. That the union between the iron and starch is at the alcoholic group seems unlikely, since such compounds are formed only in alkaline solution. The logical point of attachment is through the residual valences of the oxygen of the glucoside linkage of the starch, for at this point the splitting occurs.

In the light of these facts, it seems that the iron (or other metal), in addition to catalyzing the liberation of energy from the peroxide,

unites with the starch in a loose chemical union to pass the energy of the peroxide decomposition into the starch molecule. The energy level of the starch is thus raised, causing the starch to be reactive.

The following theory of the mechanism of starch hydrolysis is thus advanced: The iron atom forms an unstable combination with the starch, possibly through the residual valences of the oxygen of the glucoside linkage. The iron gives up its energy to the starch molecule, thus raising the energy level of the starch molecule, and making it more reactive. The iron, even in its highest energy level, does not contain enough energy to split the starch except at a very slow rate. The decomposition of the peroxide produces large quantities of energy which is taken up to form activated iron, and the energy of the iron is passed into the starch molecule. The activated starch reacts with water and is hydrolyzed. Enough energy is put into the starch molecule to cause a quite rapid hydrolysis. The iron, upon giving up the energy to the starch molecule, reverts to a lower energy level, is again activated by the peroxide, and in turn passes this energy to another molecule of starch. The reaction is a true catalysis, since the iron left is available for many transfers of energy. This is in line with Warburg's (10) suggestion that Fe^{IV} is the form of iron in its active state.

$$Fe^{II} \longrightarrow Fe^{III} \longrightarrow Fe^{IV}$$

$$Fe^{IV} + starch + H_2O \longrightarrow Fe^{II} + \begin{cases} dextrins \\ glucose \\ oxidation\ products \end{cases}$$
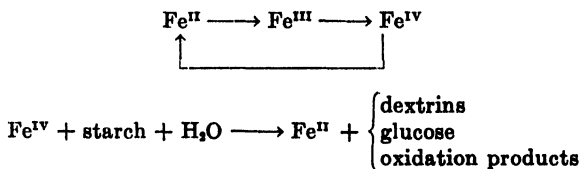
The peroxide-iron-starch system appears to be identical with the hydrolytic enzymes except for the fact that the supply of available energy is limited to the amount of peroxide present, and the system may be called an artificial enzyme.

SUMMARY

The action of hydrogen peroxide and ferrous sulfate upon starch is a hydrolysis, producing in the course of the reaction dextrins, sugars of high molecular weight, and simple sugars. The reaction is analogous to that produced by amylase, differing only

in the fact that the simple sugars produced are further hydrolyzed and oxidized to acids and aldehydes. The reaction appears to be a true catalysis, the iron acting to transfer energy from the peroxide breakdown to the starch molecule, thus raising the energy level of the starch and causing it to be reactive.

I wish to take this opportunity to express my sincere gratitude and appreciation to Dr. Albert P. Mathews for advice and assistance during the course of this study. To Mr. Charles G. Merrell I am deeply indebted for the donation of the William S. Merrell Fellowship.

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STUDIES IN HISTOCHEMISTRY

VI. THE QUANTITATIVE DISTRIBUTION OF VITAMIN C IN THE SMALL INTESTINE

By DAVID GLICK and GERSON R. BISKIND

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Our previous studies of the quantitative histological distribution of vitamin C were concerned with certain endocrine organs, *e.g.* adrenal (1), hypophysis (2), and corpus luteum (3). These endocrine glands were particularly interesting since they contained the highest concentrations of vitamin C found in the animal body. Within the last year attention has been focused upon the rôle of the small intestine in the metabolism of vitamin C.

Intestinal hemorrhages and functional disorders have long been recognized as occurring in the syndrome associated with deficiency of vitamin C. That vitamin C is involved in certain other pathological conditions of the intestine is indicated by the work of McConkey and Smith (4) who found that an adequate supply of vitamin C usually protected guinea pigs from ulcerative intestinal tuberculosis. The small intestine contains the vitamin in a comparatively high concentration, *e.g.* in about the same concentration as liver. Giroud and Leblond demonstrated that silver nitrate injected intravenously into guinea pigs was reduced by the small intestine (5). Harde and Wolff (6) reported 0.18 to 0.37 mg. of vitamin C per gm. of tissue in the mouse; Jacobsen (7) found 0.20 mg. per gm. in the normal guinea pig, while Zilva (8) recorded 0.14 to 0.30 mg. per gm. in this animal.

The stomach of the mouse contained 0.02 to 0.05 (6), that of the guinea pig 0.10 (7), and that of the rabbit 0.02 to 0.03 mg. per gm. (9). These lower values found in the stomach are likewise found in the large intestine, since this latter organ contained 0.05 to 0.12 mg. per gm. in the mouse (6) and 0.08 to 0.10 mg. per gm. in the guinea pig (7, 8).

It has been suggested by Harde and Wolff (6) that animals, such as the mouse, which synthesize their vitamin C, do this in the small intestine. The selective absorption of the vitamin from the circulation by the small intestine has been shown by Zilva (8). Hopkins (10), experimenting with rats, demonstrated changes of the concentration of vitamin C in the small intestine in fasting and with variations in the proportion of protein and carbohydrate in the diet.

Aside from extending our studies of the histological distribution of vitamin C, the present investigation extends previous work on the histological distribution of certain important constituents of the alimentary tract. Linderstrøm-Lang and Holter made a study of the distribution of pepsin (11), acid (12), and peptidase (13), in the gastric mucosa of the pig, and a study of the peptidase (13) in the duodenal mucosa. Glick investigated the distribution of esterase (14) in the stomach and duodenum of the same animal. Correlation of the chemical findings with the histology was then made in each of these cases (15).

It was the purpose of the present investigation to determine the vitamin C concentrations in serial microtome sections of fresh beef duodenum and jejunum, and to correlate, where possible, the quantitative occurrence of the vitamin C with the histological picture.

EXPERIMENTAL

Beef tissues were employed for this study so that the results might be comparable with those obtained in our other vitamin C investigations on beef tissues. The similarity in the histological structure of bovine and human intestine rendered the former quite suitable for our purposes.

It was found that duodenal or jejunal mucosa obtained from an animal killed in the morning has undergone extensive autolysis by the afternoon, even though the tissue had been kept at a temperature near $0°$. Accordingly, sections of intestine were cut open, emptied of any contents they might contain, and stretched out on a cake of solid carbon dioxide. The tissue was kept in this state until it was used, which was about 3 to 4 hours later. No discernible autolysis occurred with this technique, and the freezing of the tissue in a slightly stretched condition gave a sample of intestine in which the mucosal folds were somewhat flattened.

The sampling and sectioning of the tissue were conducted in a manner similar to that previously described (1–3). A cork borer having a diameter of 4.2 mm. was pushed through the frozen tissue at right angles to the surface, and the cylinder of tissue thus removed was fixed to the freezing block of a rotary microtome with a drop of normal saline. Sections 30 μ thick were cut and titrated individually for vitamin C with a 0.05 per cent solution of 2,6-dichlorophenol indophenol by the method previously employed (16). After titration, the sections were fixed, stained with hematoxylin and eosin, and mounted as in our previous work (1) to determine the histological character of the slices of tissue analyzed. A curve was then constructed to show variations in the vitamin C concentration through the various layers of the intestine.

No attempt was made to determine the quantity of vitamin C per cell in the mucosa, since, in this portion of the intestine, there exists a fairly homogeneous mixture of three kinds of mucosal cells in addition to various forms of leucocytes and connective tissue cells, making it impossible to be certain of the relative vitamin content of each type of cell.

However, in the Brunner's gland region of the duodenum, only one type of cell occurs, the very few connective tissue cells being disregarded; and in this region, cell counts were carried out so that the vitamin C content per cell might be estimated. Paraffin sections 6 μ in thickness were stained with hematoxylin and eosin for this purpose. These sections were prepared from formalin-fixed tissue surrounding the hole left after the cylinder of tissue had been removed for titration. The cell counts were made on these sections in the same manner as in the case of the adrenal studies (1). All whole nuclei of cells of Brunner's glands and fragments one-half or greater in size were included for the count.

Results

The histological appearance of a cross-section of the duodenum is shown in Fig. 1. The drawing has been constructed to the same scale as the abscissa so that a direct correlation might be made between the cellular nature and the vitamin C concentration at any given point.

The distribution of vitamin C in the jejunum is demonstrated in Fig. 2. There are no Brunner's glands in the jejunum, but otherwise the histology is the same as in the duodenum.

Figs. 1 and 2 are considered typical curves since each has been checked by four separate experiments on tissue from four different animals.
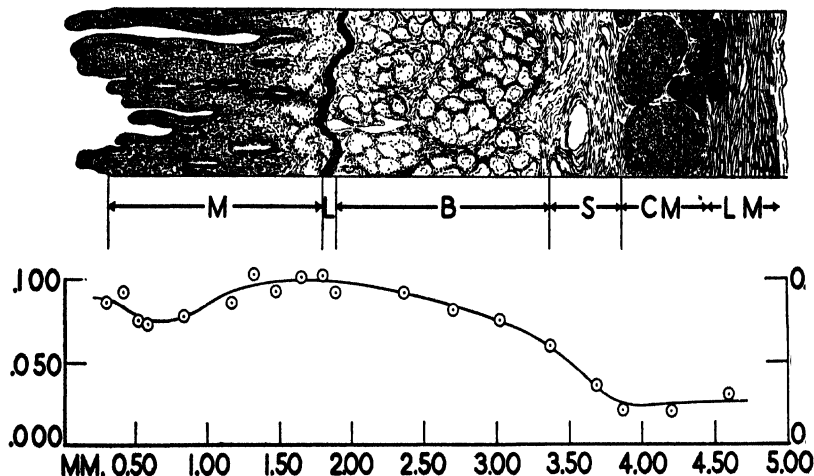
FIG. 1. Vitamin C concentrations in various portions of the duodenum. *M* represents mucosa; *L*, muscularis mucosæ; *B*, Brunner's glands; *S*, submucosa; *CM*, circular muscle; *LM*, longitudinal muscle. The left ordinate represents micrograms of vitamin C per section; the right ordinate, mg. of vitamin C per gm.
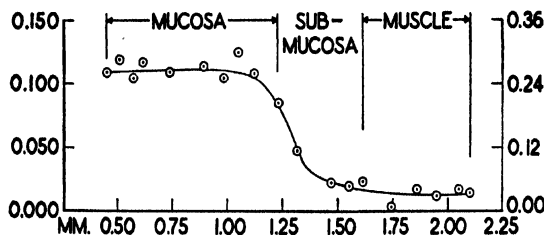
FIG. 2. Vitamin C concentrations in various portions of the jejunum. The left ordinate represents micrograms of vitamin C per section; right ordinate, mg. of vitamin C per gm.

The cell counts in the duodenum in the region of Brunner's glands are given in Table I. The total volume of the cells counted

was 0.072 $m\lambda$ of stained tissue, which was equivalent to 0.15 $m\lambda$ of fresh tissue when the latter value was calculated from the shrinkage factor 1.27. The volume of the sections titrated was 0.030 mm. $\times$ ($\pi/4$ (4.2)^2) sq.mm. = 0.415 c.mm. or 415 $m\lambda$.

TABLE I

Amount of Vitamin C and Number of Cells per Unit Volume in Brunner's Glands of the Duodenum

Cells counted in 0.072 $m\lambda$ stained tissue	58, 57, 50, 63, 66, 77, 58, 79, } 64 Average 68, 63, 74, 60
Average No. of cells per $m\lambda$ fresh tissue	426
" " " $\times 10^3$ per 30 μ slice fresh tissue	177
Micrograms vitamin C per 30 μ slice	0.085
Average micrograms $\times 10^{-6}$ vitamin C per cell	0.48

DISCUSSION

From Fig. 1 it may be seen that the vitamin C concentration is a little less in the first part of the duodenal mucosa than in the deeper portion, probably owing to the presence of crypts. The concentration stays up in the region of Brunner's glands, drops off in the submucosal layer, and falls almost to zero in the muscle. The bovine muscularis mucosæ, separating the mucosa from the Brunner's glands, is very thin (7 to 10 cells in thickness) and does not lie completely in one plane. Hence it was impossible to obtain sections of muscularis mucosæ alone, or sections which contained sufficient of this tissue to lower the titration value significantly. There can be no doubt, however, that the muscularis mucosæ like other muscle tissue, contains a very low concentration of vitamin C.

From Fig. 2 it may be seen that the vitamin C concentration is rather constant throughout the mucosa; it decreases in the submucosa and falls to a low value in the muscle just as in the case of the duodenum.

Apparently vitamin C is not localized in a specific layer of the duodenum, since the mucosa and portions including Brunner's glands both contain about the same concentration. The sub-

mucosa of both parts of the intestine is composed of fibrous tissue. As in the case of fibrous tissue in other parts of the animal body, the vitamin C concentration is low. Vitamin C is probably involved in the formation of this tissue, since Wolbach and Howe have shown that the vitamin is necessary for the production of intercellular substance (17).

SUMMARY

A histochemical study was made of the quantitative distribution of vitamin C in the duodenum and jejunum of the cow.

The mucosa and region of Brunner's glands in the duodenum contained approximately the same concentrations of vitamin C, *e.g.* 0.20 to 0.24 mg. per gm. of tissue. The submucosa was found to have on the average 0.10, and the muscle 0.06 mg. per gm.

The mucosa of the jejunum contained 0.26, the submucosa an average of 0.10, and the muscle 0.03 mg. per gm.

The region of Brunner's glands in the duodenum was estimated to possess 0.48×10^{-6} microgram of vitamin C per cell.

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AN IMMUNOLOGICAL STUDY OF THE REDUCTION OF DISULFIDE GROUPS IN PROTEINS

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During the past few years much evidence has accumulated which indicates the close dependence of the immunological behavior of proteins and their derivatives upon chemical constitution. Thus denaturation (1, 2), halogenation, nitration (3), acylation (4), and treatment with phenyl isocyanate (5) have been clearly shown to modify the immunological specificity of proteins.

Du Vigneaud and his coworkers (6) have shown that with insulin, reduction of the disulfide groups results in a complete and irreversible loss of hormone activity. It seemed, therefore, of interest to investigate the immunological effect of reduction and regeneration of disulfide groups. Horse serum albumin was chosen for this purpose because of its high content of cystine, none of which exists in the sulfhydryl form (7), and because some of its immunological relations have been worked out on a quantitative basis (8).

Similar experiments were carried out with crystallized egg albumin. This protein, in contrast to serum albumin, gives practically no color on treatment with cyanide and nitroprusside, and therefore appears to be incapable of yielding normally functioning sulfhydryl groups.

Preparation of Antigens—Crystalline serum albumin for use in this study was prepared according to Adair and Robinson (9). The reduced serum albumin was obtained by treating a solution of crystalline serum albumin with thioglycolic acid at pH 8. Freshly distilled thioglycolic acid was taken in the proportion of 1 cc. for every 25 mg. of protein nitrogen. This constitutes an excess of about 100-fold and should effect practically complete reduction of the S—S groups (10) without otherwise appreciably disturbing

the protein molecule (7, 11). The acid was first neutralized with alkali to phenol red, the protein was then added, and more alkali added if necessary to a faint red color. The flask was stoppered and set in the refrigerator overnight. The material was then dialyzed against distilled water, dialysis being continued for 16 hours after the last trace of odor had disappeared. The solution was then made isotonic with sodium chloride and an aliquot taken for nitrogen determination.

Crystalline egg albumin, prepared by the method of Sörensen and Höyrup (12) was treated with a 300-fold excess of cysteine at pH 8, in the presence of a trace of ferric chloride. Dialysis was omitted. This procedure permitted the injection of large amounts into rabbits and avoided the danger of reoxidation.

In all cases except the two indicated in Table I, the reoxidized proteins were prepared by treating the reduced products with a 10-fold excess of H_2O_2 (calculated on the basis of the total sulfur) and then making just alkaline to phenol red. This excess insures reoxidation but is insufficient to cause appreciable denaturation.

Antisera—Of the antisera to serum albumin, Antiserum 1 was obtained from a single rabbit and Antiserum 2 consisted of the pooled sera from several rabbits which had been immunized by repeated small intravenous injections of crystalline horse serum albumin.

The antisera from egg albumin, native and variously treated, were also obtained after repeated intravenous injections of small amounts of antigen.

All the sera were collected aseptically and further protected by the addition of merthiolate.

Determination of Amounts of Specifically Precipitated Nitrogen in Sera—The precipitates were prepared and their nitrogen determined according to Heidelberger, Kendall, and Soo Hoo (13). The test antigens were made up in isotonic saline. The supernatant liquids from the first centrifugation were divided into three parts and tested for excess of antigen with serum, and for excess of antibody with both native and reduced or reoxidized antigens.

The results of the quantitative determinations are presented in Tables I and II.

Serum Albumin—With the homologous antigen the supernatant liquids, as expected, gave positive tests for residual antibody

up to the point of maximum precipitation, and for residual antigen from there on.

TABLE I

Precipitin Reactions with Egg Albumin

Antiserum to egg albumin	Maximum total N precipitated with 1 cc. serum by egg albumin			
	Native	Reduced	Reoxidized	Heat-denatured
	mg.	*mg.*	*mg.*	*mg.*
Native; Sample 1..............	0.806	0.788	0.781	0.092
" " 2..............	0.536	0.524	0.529	0.073
Reduced; " 1..............	2.244	2.094	2.274	0.206
" " 2..............	1.446	1.546	1.498	0.190
Reoxidized.......................	0.260	0.254	0.268	0.152
Denatured; Sample 1............	0.086	0.086	0.154*	0.304
" " 2............	0.334	0.314	0.596*	1.780

' 100-fold excess of H_2O_2 was employed for reoxidation.

TABLE II

Precipitin Reactions with Antisera to Horse Serum Albumin

Antiserum No.	N antigen	Total N in precipitate with 1 cc. serum and					
		Native serum albumin (a)	(a) reduced (b)	(a) treated with H_2O_2 (c)	(b) reoxidized (d)	Supernatant from (b) treated with (a)	Supernatant from (d) treated with (a)
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.02	0.202					
	0.04	0.402					
	0.06	0.502					
	0.08	0.534					
	0.10	0.576	0.430			0.064	
	0.12	0.499	0.441			0.017	
	0.15		0.444				
	0.20	0.459	0.474			0.010	
2	0.04	0.451					
	0.06	0.528	0.184	0.537	0.162	0.031	0.022
	0.08	0.510	0.222	0.519	0.216	0.016	0.012
	0.15	0.440		0.435			
	0.165		0.263		0.267	0.009	0.005

The effect upon the precipitin reaction of reduction and reoxidation reveals itself in two distinct ways. Firstly, the absolute

amount of nitrogen precipitated is definitely less in the cross-reaction than in the homologous precipitin determination. It may be pointed out that qualitative precipitin tests would scarcely have yielded this information. Secondly, the usual inhibition of precipitation by excess of test antigen is not found; on the contrary, there is a slight but steady increase of precipitate as the excess of antigen is increased.

All the supernatant liquids from precipitates in the cross-reactions between reduced or reoxidized serum albumin and antisera to native albumin gave positive tests for residual antibody when treated with native albumin. Attempts to precipitate the remaining antibody, however, by adding a calculated amount of native antigen to an aliquot of the supernatant liquid from the first centrifugation yielded only small amounts of precipitate which diminished with excess of heterologous antigen, much as was found by Heidelberger and Kendall (14) in a quantitative study of another cross-reaction.

As a control, native serum albumin was treated with hydrogen peroxide. The protein was not affected immunologically.

Egg Albumin—No notable differences in immunological behavior between the native, "reduced," and "reoxidized" forms of egg albumin could be detected (Table I). Heat denaturation by the method of Wu, Ten Broeck, and Li (1), however, caused a marked difference, already observed qualitatively by these investigators and others (15, 16). The high values observed on adding "reoxidized" egg albumin to antidenatured egg albumin serum may be ascribed to partial denaturation (17) by the 100-fold excess of hydrogen peroxide employed in these experiments.

With egg albumin, the failure of treatment with cysteine to induce immunological differences correlates with the failure of the cyanide-nitroprusside test for sulfhydryl; on the other hand, the difference observed with heat-denatured egg preparations correlates with the appearance of sulfhydryl groups on coagulation by heat (18).

These results indicate either that the S—S and —SH groups may be among the specific chemical groups involved in antigen-antibody combination, or that the reduction of the S—S linkage may split the antigen molecule into units which are too small to give the same immunological response as the original protein.

In conclusion the writer wishes to thank Dr. Hans T. Clarke and Dr. Michael Heidelberger for their interest and help.

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PATHS OF EXCRETION AND MINERAL BALANCE IN ANIMALS DRINKING SALINE AND ALKALINE WATERS

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During the past 8 years this station has conducted many experimental tests to determine the effect on animals of consuming drinking waters which contained various salts and alkalies in solution. It was found (1) that, when the concentrations were increased above certain percentages, the animals could not continue to grow and reproduce as normal animals; with waters below these concentrations and still quite saturated, the animals seemed able to utilize or to eliminate the excess with no apparent effort. Studies were also made and reported (2) showing that there was little increase in the mineral content of the blood or body serums and that such changes came only with the approach of death of the animal.

As a result of these findings, the question naturally arises as to what paths of excretion are followed by the large quantities of salts ingested, and what effect these inorganic ions would have upon the mineral balance of the body. A review of literature reveals so many references to various aspects of the subject that only a few may be mentioned. McCrudden (3), Nelson and Burns (4), Bogert and McKittrick (5), Richards, Godden, and Husband (6), Leiter (7), and Fine (8) have conducted experiments with calcium excretion and show that there are considerable variations in the feces-urine distribution and in retention, depending upon the animal's age, nature of ration, and other conditions. Lebensohn (9), Baumann and Oviatt (10), and Richards and coworkers (6) state that regardless of other factors the bulk of the chlorides is usually found in the urine. The reports of Baumann and Oviatt (10), Hele (11), and von Noorden (12) show considerable varia-

tions in regard to the paths of sulfate excretion. A similar statement may be made for other ions and in no case do we find any definite answer to the cause of the difficulties arising from the ingestion of excessive amounts of inorganic ions in the drinking water. An attempt to answer these questions suggested the experiment, the results of which are here recorded.

Procedure

Mineral balances have been conducted with cattle, hogs, guinea pigs, and rats at all seasons of the year, and over a 6 year period. Because the rat can be handled more quickly, and the results are the most accurate, data from that source are reported here. In all cases healthy stock from our colony have been used and housed in sanitary metal cages. The drinking water was either distilled or a solution of the indicated salt added to the distilled water. The basal feed consisted of cereal grains, supplemented with animal proteins, minerals, and vitamins in the proper proportions. The record of growth, food intake, and water consumption was recorded for several weeks or months prior to the time the experiments were conducted. During the experimental periods the animals were confined to lacquered wire cages, fed from specially constructed feeders from which the feed could not be wasted, and the actual daily intake was calculated. The drinking solution was given from automatic fountains which likewise permitted accurate measurement. Beneath the wire floor was a second glass screen on which the feces were collected, while beneath this screen the urine was gathered in large evaporating dishes. In most of the experiments four rats were used and samples taken for 3 day periods. The following week the experiments were repeated until checks were obtained. The methods used for the analysis of feces, urine, feed, and waters were all standard procedures. The feces and feeds were ashed, as suggested by Tisdall and Kramer (13). The calcium and magnesium in the feed, feces, and urine were determined by McCrudden's method (14). Chlorides were obtained in water and urine by a modified Volhard-Harvey procedure (15) and in the feed by the Van Slyke and Sendroy method (16). The phosphorus was determined by slight modification of Neumann's procedure (17); the sulfates, by the Benedict technique (18).

By these methods, samples of feed, feces, drinking waters, and urine have been analyzed repeatedly from the various groups and converted into terms of total mg. intake and output per 12 rat-day periods.[1]   In every case the figures in Tables I to V represent not one determination, but the average of three or more sets of figures obtained in successive trials.   In all biological tests there are always some unavoidable variations from day to day, but if the trend of the various individual sets of data in these experiments were not similar, the entire series was repeated and the averages computed.

The chloride studies of such a series are recorded in Table I. The chloride content of the food and drinking water consumed by four rats during a 3 day period, as well as the quantity excreted during a similar period, is recorded in mg. per 12 rat-day periods. From these figures the chlorine balances and the percentages of the same in the urine and feces have been calculated.   The data in Table I, Series A, were for mature rats in the cool spring weather.   Series B represents similar trials during rather hot weather of the late summer, and Series C for younger growing animals in the early summer.   The drinking waters, in all cases, were prepared from distilled water and pure salts.   The basic ration, prepared as previously described, was kept constant throughout a given series of tests but was altered somewhat in Series A, B, and C.

These data indicate that increasing the chloride intake increases the chloride storage.   The path of excretion is also somewhat altered as the percentage in feces increases with the intake, indicating that the absorption is somewhat altered, as might be expected in the changing osmotic pressures.   The presence of sulfates causes an increased output in the feces, probably owing to delayed absorption as well, causing a cathartic action.

It was also an interesting observation that sodium chloride in the drinking water seemed to stimulate, while calcium chloride and calcium hydroxide inhibited, food consumption.

Table II represents the data for sulfur metabolism taken from the same group of animals used for the chloride determinations, the results of which are recorded in Table I, Series C, the method of

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[1] These were periods of 3 days during which experiments were carried out upon four rats.

TABLE I

Chlorine Ingested and Excreted in a 12 Rat-Day Period

Series	Ration*	Chloride intake			Chloride excretion			Chloride balance
		Water	Feed	Total	Total	In feces	In urine	
		mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*
A	1.5% MgSO₄ in water	0	945	945	784	9.0	91.0	+161
	1.5% NaCl " "	3217	936	4153	3818	5.8	94.2	+335
	1.5% CaCl₂ " "	1438	678	2116	1427	6.4	94.6	+689
	Saturated Ca(OH)₂-water	0	1038	1038	796	8.9	91.1	+242
	" CaSO₄-water	0	1111	1111	846	13.7	86.3	+265
	5% NaCl in feed and distilled water	0	4758	4758	4041	9.8	90.2	+717
	5% CaCO₃ in feed and distilled water	0	936	936	607	6.8	93.2	+329
B	Distilled water	0	1293	1293	1024	18.1	81.9	+269
	1.5% NaCl in water	4129	1520	5649	5361	9.1	90.9	+288
	1.5% CaCl₂ " "	2550	1235	3785	3375	8.8	91.2	+410
	1.5% MgSO₄ " "	0	1424	1424	1143	26.6	73.4	+281
C	Distilled water	0	1482	1482	1227	14.1	85.9	+255
	Saturated Ca(OH)₂-water	0	1273	1273	958	14.9	85.1	+315
	" CaSO₄-water	0	1742	1742	1298	12.6	87.4	+444
	1.5% CaCl₂ in water	1780	1381	3161	2300	8.3	91.7	+861
	1.5% MgSO₄ " "	0	1470	1470	1151	16.7	83.3	+320

* The basal ration was common, the drinking water, or feed, being supplemented as indicated.

TABLE II

Sulfur Balance and Paths of Excretion in a 12 Rat-Day Period

Ration*	S intake			S excretion			S balance
	Water	Feed	Total	Total	In feces	In urine	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*
Distilled water	0	197	197	183	62.3	37.7	+14
1.5% NaCl in water	0	231	231	194	55.1	44.9	+37
1.5% MgSO₄ " "	1226	217	1443	822	38.9	61.1	+621
1.5% CaCl₂ " "	0	188	188	166	81.2	18.8	+22

* The basal ration was common, the drinking water, or feed, being supplemented as indicated.

feeding, collecting, and handling samples being the same as for that series.

There have always been numerous questions in regard to sulfur metabolism and elimination.   This problem is more complicated, as we are dealing with an ion found in both the inorganic and organic forms, free or conjugated.   The data bear out these theories;

TABLE III

Effects of Various Concentrations of Calcium Salts in Water upon Path of Excretion of Calcium Ions in a 12 Rat-Day Period

Series	Ration*	Ca intake			Ca excretion			Ca balance
		Water	Feed	Total	Total	In feces	In urine	
		mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*
A	Distilled water	0	1730	1730	1620	98.7	1.3	+110
	Saturated Ca(OH)₂-water	199	1392	1591	1424	98.0	2.0	+167
	" CaSO₄-water	151	1722	1873	1784	98.2	1.8	+89
	1.5% CaCl₂ in water	1100	1897	2997	2722	97.0	3.0	+275
	12% CaCO₃ " feed and distilled water	0	5152	5152	5130	98.0	2.0	+22
	3% CaCO₃ in feed and distilled water	0	1813	1813	1696	98.0	2.0	+115
B	Distilled water	0	1688	1688	1369	99.1	0.9	+322
	Saturated Ca(OH)₂-water	124	1440	1564	1372	98.6	1.4	+192
	" CaSO₄-water	127	1975	2102	1768	98.0	2.0	+334
	1.5% CaCl₂ in water	827	1567	2394	1743	96.9	3.1	+651
	1.5% MgSO₄ " "	0	1668	1668	1566	98.3	1.7	+102
C	Distilled water	0	1529	1529	1294	99.5	0.5	+235
	1.5% NaCl in water	0	1796	1796	1460	98.6	1.4	+336
	1.5% MgSO₄ " "	0	1683	1683	1395	97.2	2.8	+288
	1.5% CaCl₂ " "	1439	1459	2898	2283	94.9	5.1	+615

* The basal ration was common, the drinking water, or feed, being supplemented as indicated.

the path of excretion is not constant, depending upon the quantity of intake and the accompanying ions.   If the ion be one that forms insoluble sulfates such as calcium, then the percentage in the feces is increased; on the other hand, the presence of sodium chloride causes a greater portion to be eliminated in the urine.

Table III, Series A, B, C, presents data showing the effect of various concentrations of different salts upon the balance and ex-

cretion of the calcium ion.   The feces are recognized as the normal path of calcium excretion.   Even calcium compounds from the blood are thought to be reabsorbed into the intestinal tract in part and so eliminated.   This peculiar method of intestinal excretion makes it difficult to postulate the true course of calcium through the body and its real significance.   If the calcium is consumed in the form of chloride or in company with ions favoring the formation of chlorides, the absorption is evidently increased and the excretion in the urine is greater; however, the kidney is always of small importance in its elimination.

<div align="center">TABLE IV</div>

Mineral Balance and Paths of Excretion of the Magnesium Ion in a 12 Rat-Day Period

Series	Ration*	Mg intake			Mg excretion			Mg balance
		Water	Feed	Total	Total	In feces	In urine	
		mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*
A	Distilled water	0	317	317	229	89.1	10.9	+88
	1.5% NaCl in water	0	373	373	240	91.3	8.7	+133
	1.5% MgSO₄ " "	963	355	1319	580	83.8	16.2	+739
	1.5% CaCl₂ " "	0	303	303	409	88.1	11.9	−106
B	Distilled water	0	482	482	229	94.9	5.1	+253
	Saturated Ca(OH)₂-water	0	413	413	250	81.3	18.7	+163
	1.5% CaCl₂ in water	0	441	441	413	91.3	8.7	+28
	1.5% MgSO₄ " "	718	477	1195	621	85.1	14.9	+494

* The basal ration was common, the drinking water, or feed, being supplemented as indicated.

The data for magnesium are given in Table IV, Series A and B. Investigators have drawn the conclusion that magnesium resembles calcium in its functions and in its elimination.   The data here presented do not confirm that conclusion, but possibly the fact that we are dealing with large intakes of each alters the condition.   It is true that the mass of the ion does find its path of elimination through the feces, but not to the extent found for calcium.   For reasons not explained, the presence of the sulfate ion seems to increase magnesium and inhibit calcium elimination in the urine.   The data for Series A and B in Table IV are for rats approaching maturity during the warm summer weather.

The basic rations for the two were somewhat different. The peculiar method of intestinal excretion makes it difficult to postulate the real course of calcium through the system.

Table V, Series A, records the data for the phosphorus studies of groups of actively growing rats during the warm summer weather, and Series B for mature rats during the colder weather. The growth of the latter lot was less than that of those reported as

TABLE V

Mineral Balance and Paths of Excretion of the Phosphorus Ion in a 12 Rat-Day Period

Series	Ration*	Phosphate intake			Phosphate excretion			Phosphate balance
		Water	Feed	Total	Total	In feces	In urine	
		mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*
A	Distilled water	0	800	800	681	95.25	4.75	+119
	Saturated Ca(OH)₂-water	0	687	687	672	96.79	3.21	+16
	" CaSO₄-water	0	940	940	822	94.55	5.45	+118
	1.5% CaCl₂ in water	0	746	746	654	98.25	1.75	+92
	1.5% MgSO₄ " "	0	794	794	664	98.58	1.42	+130
B	1.5% Na₂HPO₄ in water	851	1005	1856	1592	70.40	29.60	+264
	1.5% MgSO₄ " "	0	741	741	725	91.80	8.20	+16
	1.5% NaCl " "	0	730	730	676	83.88	16.12	+54
	1.5% CaCl₂ " "	0	532	532	520	90.76	9.24	+12
	Saturated Ca(OH)₂-water	0	815	815	742	91.24	8.76	+73
	" CaSO₄-water	0	880	880	802	90.40	9.60	+78
	Basic ration, 5% NaCl, and distilled water	0	825	825	746	84.50	15.50	+79
	Basic ration, 5% CaCO₃, and distilled water	0	734	734	772	92.17	7.23	−39
	Distilled water	0	725	725	685	90.47	9.53	+40

* The basal ration was common, the drinking water, or feed, being supplemented as indicated.

Series A. It will be noted that the phosphorus balance is increased by the high phosphorus feeding, and the percentage eliminated through the urine is also increased.

Increasing the calcium or magnesium ion increases the fecal phosphorus output. It will be noted that the kidney plays the principal rôle in taking care of changing conditions when the body is under particular stress.

SUMMARY AND CONCLUSION

An increase of the mineral content of the drinking water to the maximum amount which will not cause serious injury to the animal produces an abnormal mineral content of the urine and feces and at the same time alters the normal paths of excretion.

Quantitative studies, over a period of several years, demonstrate that there are definite trends repeating themselves so regularly that certain conclusions may be stated.

1. About 90 per cent of the chlorides is excreted through the urine, though the percentage found in the feces of these animals is much in excess of the amount normally reported; especially is this true when the intake is increased or when acid-producing ions accompany the chlorides in the drinking water. The increase in percentage of fecal chlorides is probably a result of the change in osmotic pressure caused by the increased salt content of the alimentary tract and the resulting cathartic action. Invariably, chlorine in the form of calcium chloride causes the greatest chloride retention.

2. Sulfur is excreted in approximately equal amounts in the feces and urine, the path depending upon the quantity and form ingested. The presence of an ion with which the sulfates form insoluble compounds increases the fecal phosphorus output. Increasing the sulfur intake increases the sulfur retention as well as the percentage in the urine.

3. The retention of calcium in the body corresponds somewhat with the amount consumed. Calcium in the form of the chloride seems to be the most favorably absorbed, producing a more positive balance as well as increasing the percentage found in the urine. In no case has the percentage found in the urine equaled the amounts often reported by others. Under conditions of abnormal mineral intake the body must depend upon the feces to carry away the greater portion of the quantity consumed.

4. Magnesium is said to parallel the action of calcium in its reaction. It has been found that more than 10 per cent is eliminated in the urine as compared to less than 3 per cent of the calcium. Excessive amounts of calcium seem to displace magnesium from the body.

5. The phosphorus in these studies is found in the feces in greater amounts than previously reported; especially is this true

when large amounts of calcium or magnesium salts are present. Increasing the phosphates in the feed or water causes a sudden increase in the retention and the percentage excreted in the urine.

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STUDIES ON ALUMINUM

I. A CRITICAL STUDY OF QUANTITATIVE COLORIMETRIC METH-
ODS FOR ALUMINUM ON BIOLOGICAL MATERIAL*

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Within the past few years application of colorimetric methods
has made possible the quantitative estimation of a number of
mineral elements including aluminum in biological material. In
most of these methods the aluminum is determined by the color
resulting from combination of the aluminum with certain dyes.
The formation of the aluminum-aurin lake, as it applies to colori-
metric procedures, has been investigated by Yoe and Hill (1),
Myers, Mull, and Morrison (2), Winter, Thrun, and Bird (3), and
Cox and his associates (4). These investigators have found that in
the presence of excess dye in acid solution a rather stable color
compound of aluminum and aurin is formed and the excess dye
may be decolorized at pH 7.0 to 7.3 without decomposing the lake.
Atack (5), Yoe and Hill (6), and Underhill and Peterman (7)
have investigated the use of alizarin to form the color lake of
aluminum for a basis of colorimetric estimation. Kolthoff (8)
has described a very sensitive color test for aluminum by use of
1, 2, 5, 8-hydroxyanthraquinone. Teitelbaum (9) precipitated
the aluminum as the salt of 8-hydroxyquinoline and then estimated
the aluminum by the amount of color formed by subjecting the

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quinoline to the Folin-Denis phenol reagent. Myers, Mull, and Morrison (2), Winter and Bird (10), and Cox *et al.* (4) have described methods of separating aluminum from interfering elements, based on the solubility of the aluminate ion in sodium hydroxide. Underhill and Peterman (7) separated the soluble thiocyanates from tissue ash solutions by means of ether. Philip and Brandey (11) have shown that there is danger of reducing ferric to the ether-insoluble ferrous iron in such a mixture. Cupferron has been used by Cunningham (12), Heller and Burke (13), and Cox and his associates (4) for the removal of iron from aluminum-containing solutions.

The results obtained by different workers, using the same or different methods of analyzing biological material for aluminum, have indicated that either a great variation exists in different tissues or the methods of analysis are not entirely adequate. It is for this reason that the methods have again been studied. Different colorimetric methods of determining aluminum have been compared, with the same and different methods of preparing the aluminum solutions free from interfering substances.

<div align="center">EXPERIMENTAL</div>

The following procedure has been developed for estimating aluminum in a solution free from any interfering material.

<div align="center">*Estimation of Aluminum with Aurin*</div>

Reagents

1. 1:1 dilution of ammonium hydroxide.
2. 1:1 dilution of hydrochloric acid.
3. Saturated solution of ammonium acetate.
4. Approximately 1.5 N ammonium carbonate.
5. 0.1 per cent solution of aurintricarboxylic acid prepared by dissolving 1 gm. of the aurin in 1 liter of distilled water containing 2.5 cc. of concentrated ammonium hydroxide. The aurin should be kept in a brown bottle.
6. Aluminum standard solution prepared by dissolving 3.5018 gm. of recrystallized $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$ in 1 liter of approximately 0.1 N hydrochloric acid. This stock solution is diluted 1:20 with 0.1 N hydrochloric acid to give a solution containing 0.01 mg. of aluminum per cc.

7. Congo red paper prepared by saturating ashless filter paper with an alcoholic solution of Congo red dye. After the paper is dry it is cut into pieces about 1 cm. long by 0.5 cm. wide and kept in a stoppered bottle.

Modified Klett Colorimeter—A colorimeter with a reading scale of 0 to 150 mm. was especially constructed for use in this investigation.

Procedure—Transfer the sample to a 50 cc. volumetric flask, and add 5 cc. of a 1:1 dilution of ammonium hydroxide and a piece of Congo red indicator paper. Add slowly, with constant shaking, enough approximately 6 N hydrochloric acid just to turn the indicator violet. Add 2 cc. of a saturated solution of ammonium acetate and 2 cc. of a 0.1 per cent solution of aurin, wash the neck of the flask with water, and whirl to mix the contents. Place the flask in water at 80–90° for 10 minutes, cool to room temperature, and dilute with water to about 40 cc. Add, with shaking, enough 1.5 N ammonium carbonate to change the pH of the solution to 7.2. The amount of ammonium carbonate necessary is determined on blank solutions containing no aurin and compared with a phosphate buffer solution, phenol red being used as the indicator. After the addition of the carbonate solution the flask is made up to volume and the contents are thoroughly mixed. A standard containing a definite amount of aluminum is prepared in exactly the same manner as the unknown. After standing 15 minutes to allow for the decolorization of the excess dye, the two solutions are compared in a colorimeter.

The method of Underhill and Peterman (7) has been used in all of the determinations in which alizarin has been employed. This method has been found to be quite satisfactory with pure solutions of aluminum salts.

Several modifications of the Teitelbaum (9) method for estimating aluminum have been made. The Folin-Ciocalteu (14) phenol reagent was substituted for the original Folin reagent and was found to give more accurate results. Later investigations of colorimetric estimation of 8-hydroxyquinoline compounds have indicated that not all samples of this material are the same. In this work very good color comparisons were obtained. With a later sample of 8-hydroxyquinoline secured from the same source colorimetric comparisons were very unsatisfactory.

Dr. Kolthoff kindly furnished a sample of 1, 2, 5, 8-hydroxyan-thraquinone which was used in a few determinations.   Since little correlation was found between color intensity and aluminum concentration, this reagent was used only for qualitative tests.   The procedure described by Kolthoff (8) was employed in qualitative tests.

The methods of developing the colored solutions described above have been applied in a study of the color curves and effects of certain interfering substances on the determination of aluminum by the several methods.

TABLE I

Relation of Aluminum Concentration to Color Intensity of Aurin, Alizarin, and 8-Hydroxyquinoline Color Complexes

Al present	Al found		
	Aurin	Alizarin	8-Hydroxyquinoline
mg.	*mg.*	*mg.*	*mg.*
0.0000	0.0000	Very pale	
0.0010	0.0015	0.0016	
0.0020	0.0020*	0.0024	0.0019
0.0030	0.0029	0.0030*	0.0030*
0.0040	0.0038	0.0038	0.0040
0.0025	0.0034	0.0030	0.0024
0.0050	0.0050	0.0057	0.0051
0.0075	0.0075*	0.0075*	
0.0100	0.0100	0.0095	0.0097
0.0150	0.0150	0.0120	0.0150*
0.0200	Turbid	Ppt.	0.0196
0.0250	"	"	0 0240

* Standard used for all determinations in the particular series.

In Table I are shown the color curves of the aluminum complexes of aurin, alizarin, and 8-hydroxyquinoline.   It will be seen that for aurin and 8-hydroxyquinoline a pure color holds over considerable range.   With the alizarin the standard must be very nearly the same as the unknown.   1, 2, 5, 8-Hydroxyanthraquinone determinations are not included, since the color complex tends to precipitate very readily and the method appears to be much better adapted to qualitative work.

The effects of added iron to the aurin and alizarin lakes are

shown in Table II.   With 8-hydroxyquinoline the iron is additive
to the aluminum, since both iron and aluminum form pure com-
pounds with the quinoline.   0.02 mg. of Fe is equal to approxi-
mately 0.01 mg. of Al.

It was found that, whenever solutions of iron and aluminum
were separated with sodium hydroxide and tested with alizarin,

TABLE II

Effect of Iron on Aluminum Lakes of Aurin and Alizarin

Al present	Fe present	Al found	
		Aurin	Alizarin
mg.	mg.	mg.	mg.
0.0100	0.0000	0.0100	0.0100
0.0100	0.0005	0.0118	0.0100
0.0100	0.0007	0.0127	0.0105
0.0100	0.0010	0.0145	0.0110
0.0100	0.0025	0.0190	0.0125
0.0100	0.0040	0.0240	0.0160
0.0100	0.0050	Purple	Ppt.
0.0100	0.0100	Ppt.	"
0.0100	0.0150	"	

TABLE III

Effect of Sodium Chloride on Aluminum Lakes of Aurin and Alizarin

Al present	NaCl present	Al found	
		Aurin	Alizarin
mg.	mg.	mg.	mg.
0.0100	0	0.0100	0.0100
0.0100	100	0.0114	0.0091
0.0100	200	0.0127	0.0070
0.0100	300	0.0135	0.0066
0.0100	400	0.0148	0.0048
0.0100	500	0.0160	0.0043

low values were obtained.   The effects of sodium chloride are
shown in Table III.   The increasing amount of aluminum found
with aurin appears to be due to aluminum in the salt used, since
solutions containing NaCl alone gave qualitative tests for alu-
minum with other reagents.   "Salt" effects were equally great
when the alizarin determinations were made in alcohol.

Phosphates tend to inhibit the formation of the aurin lake but do not affect the determination of aluminum by alizarin, 1, 2, 5,- 8-hydroxyanthraquinone, or 8-hydroxyquinoline.   Some experiments have indicated that ammonium molybdate would prevent the inhibition of the formation of the aurin lake by phosphates. Small amounts of calcium do not interfere with aurin or 8-hydroxyquinoline, but in the presence of calcium, aluminum and alizarin give slightly more color than in the absence of calcium.   Magnesium appears to have very little effect on the aurin lake, slightly greater on the alizarin, and to form a definite compound with 8-hydroxyquinoline.

Separation of Aluminum and Other Metals, Particularly Iron

Three methods used for the separation of aluminum from other metals which have been reported present in animal tissue have been modified so as to be applicable to tissue analysis.

Precipitation of Iron and Aluminum As Phosphates

Reagents

1. 1:1 dilution of ammonium hydroxide.
2. Saturated solution of ammonium acetate.
3. Solution of diammonium phosphate containing 20 mg. per cc.
4. Ferric chloride solution containing 1 mg. of Fe per cc.
5. Methyl red indicator.
6. Methyl orange indicator.

Procedure—The acid solution to be analyzed is transferred to a 50 cc. conical centrifuge tube.   Unless the sample is known to contain iron and sufficient phosphorus to combine with both the iron and aluminum, it is best to add 1 mg. of iron and from 50 to 100 mg. of diammonium phosphate to the solution.   1 cc. of a saturated solution of ammonium acetate and a few drops of methyl orange indicator are added to the solution, then with constant shaking or stirring ammonium hydroxide is added until the indicator just turns yellow.   A drop of methyl red indicator added to the tube should show faintly pink.   The contents of the tube are then diluted to about 25 to 30 cc. and heated in boiling water for 1 hour.   The sides of the tube are washed with a few cc. of distilled water and the tube centrifuged at high speed for 10 minutes.   The supernatant fluid is immediately decanted.   Any insoluble mate-

rial such as silica is contained in the precipitate of iron and aluminum. The removal of this material is easily effected by dissolving the phosphates in hydrochloric acid and centrifuging out the silica. Washing the residue with dilute acid removes the aluminum quantitatively. When the sodium hydroxide method of separating

TABLE IV

Comparative Qualitative Determinations on Supernatant Fluid after Precipitation of Iron and Aluminum As Phosphates

Solution No.	Constituents	Present	Aurin	Alisarin
		mg.		
1	Aluminum	0.01		
	Iron	1.00	Negative	Negative
	Phosphorus	2.00		
2	Aluminum	0.01		
	Iron	1.00	Negative	Positive
	Phosphorus	2.00		
	Manganese	0.05		
3	Aluminum	0.01		
	Iron	1.00	Negative	Positive
	Phosphorus	2.00		
	Cobalt	0.05		
4	Aluminum	0.01		
	Iron	1.00	Negative	Negative
	Phosphorus	2.00		
	Calcium	0.05		
5	Aluminum	0.01		
	Iron	1.00	Positive	Positive
	Phosphorus	2.00		
	Chromium	0.05		
6	Liver digest*		Negative	Positive
7	Blood " *		"	"

* 5.0 gm. of fresh tissue.

iron and aluminum is used, the silica is of no consequence, since it is separated out with the ferric hydroxide precipitate.

The method of precipitating the iron and aluminum as the phosphates has the advantage over the precipitation as the hydroxides in that at the pH of this procedure calcium phosphate is soluble.

Data have been presented in Table IV which show that in pure

solutions of iron and aluminum, precipitated with a slight excess of phosphate, the precipitation is quantitative.   Only a slight excess of phosphate was used, since too much phosphate would inhibit the formation of the aurin-aluminum lake in the supernatant fluid.

For tissue analysis a large excess of phosphate is used to combine with both the iron and aluminum, as ferric phosphate is more insoluble than aluminum phosphate.

Sodium Hydroxide Separation of Iron and Aluminum

In the experiments to be reported here an unpublished modification of the method of the Myers, Mull, and Morrison (2) by Mull has been used to separate the iron and aluminum.

Reagents

1. 6 N hydrochloric acid.
2. 6 N sodium hydroxide (aluminum-free).
3. Glacial acetic acid.

Procedure—The aluminum and iron are precipitated by the method described.   The precipitate is then dissolved in 1 cc. of 6 N hydrochloric acid and diluted to 15 cc.   1 cc. of glacial acetic acid is next added and the tube whirled to mix.   This is followed by 5 cc. of 6 N sodium hydroxide, after which the tube is shaken. The tube is left for 30 minutes and then centrifuged for 10 minutes. The supernatant fluid is decanted into a clean centrifuge tube. 0.25 cc. of acetic acid is then added and the tube placed in the ice box overnight, after which the tube is centrifuged and the fluid decanted into a 50 cc. Nessler tube or volumetric flask.   The color is developed by the method described for aurin.

Thiocyanate and Ether Separation of Aluminum and Iron

Reagents

1. 60 per cent solution of ammonium thiocyanate.
2. Ether containing 3 to 4 per cent alcohol.

Procedure—The acid solution is placed in a 30 cc. separatory funnel and 2 to 3 cc. of the ammonium thiocyanate solution are added.   The funnel is immediately filled with ether, stoppered, and shaken.   As soon as the two phases have separated, the stopper is removed and the ether syphoned off.   The extractions with ether are repeated until both the aqueous and ether phases are colorless.   If the sample is very high in iron, it is advisable to

add a few drops of nitric acid and more of the thiocyanate solution after the first extraction. Table V shows that manganese, cobalt, and chromium are not removed in the ether extraction.

TABLE V

Qualitative Tests on Acid Solutions of Certain Metals after Extraction with Thiocyanate and Ether

Solution No.	Constituents	Present	Alizarin
		mg.	
1	Iron	1.00	Negative
2	Aluminum	0.01	Positive
3	Iron	1.00	"
	Aluminum	0.01	
4	Iron	1.00	Negative
	Copper	0.05	
5	Iron	1.00	Positive
	Manganese	0.05	
6	Iron	1.00	Positive
	Cobalt	0.05	
7	Iron	1.00	Positive
	Chromium	0 05	

Cupferron Separation of Iron and Aluminum

Reagents

1. Concentrated sulfuric acid.
2. Concentrated nitric acid.
3. 9 per cent cupferron solution.
4. 1:1:1 mixture of benzene, ether, and acetone.

Procedure—The iron and aluminum are precipitated as the phosphates by the method previously given. The precipitate is dissolved in 0.5 cc. of concentrated sulfuric acid and enough water to insure solution of the precipitate. The solution and washings, totaling about 15 cc., are transferred to a 30 cc. separatory funnel. After the solution has cooled to room temperature, enough 9 per cent cupferron solution is added to precipitate the iron and form a white precipitate. After 5 minutes, 15 cc. of a mixture of equal parts of benzene, ether, and acetone are added, the funnel is stoppered, and the mixture is vigorously shaken. The two phases quickly separate and the aqueous phase containing the aluminum is drawn off into a Pyrex digestion tube calibrated at 50 cc. Sev-

eral 2 cc. portions of water are shaken in the funnel and also drawn off to insure complete removal of the aluminum.

The excess cupferron in the aluminum-containing solution is ashed by heating over a microburner. A drop of concentrated nitric acid added to the tube after the contents are charred hastens complete combustion. The sides of the tube are washed with distilled water and the contents boiled down until fumes of sulfuric acid come off. It is essential that all of the cupferron be removed.

Analysis of Biological Material

Preparation of the Sample—The aluminum content of animal tissue, milk, or urine is so small in comparison to the other mineral constituents that a large sample is required for analysis. 50 gm. of tissue are ashed when that much is available; while for urine and milk much larger samples are required if duplicate determinations are to be made.

Tissue samples are dissected out with nickel scissors or a scalpel and washed with distilled water to remove any adhering blood or hair. The water is allowed to drain away by placing the tissue in a glass funnel for a few minutes. The samples are then placed directly into weighed silica crucibles and the weight of tissue determined. Milk and urine samples should be collected in Pyrex flasks and weighed into 200 cc. silica evaporating dishes. The samples are then placed in an electric oven overnight in order to drive off as much water as possible. Milk and urine should be evaporated to dryness.

Ashing—Ashing is begun by heating the silica container, supported on a triangle, with a Bunsen burner. This quickly chars the organic material and hastens the ashing process. The preliminary heating must be done very carefully as the material swells and will overflow the container unless the flame is occasionally removed to allow for cooling. When the charred material has ceased to foam on further heating, it may be placed in the muffle oven without danger of loss by overflowing or spattering. The temperature of the muffle oven must not be over 500°. If too high a temperature is used, the ash will fuse into the silica and cannot be dissolved out with strong acid. There is also loss of aluminum at high temperatures, probably owing to fusion of the ash. The door of the oven should be left slightly ajar in order that adequate

oxygen is available for the complete combustion of the carbon. Small particles of carbon may not be ashed by this procedure, but they will not interfere as the acid used later in the digestion will completely ash the residue.   Usually 24 hours is sufficient time for the organic material to be completely decomposed and a light fluffy ash left.   The ash and any remaining carbon is digested with a mixture of equal parts of nitric and sulfuric acids.   10 cc. of the acid mixture are used for 50 gm. of tissue.   The acid is added to the cold silica dish after the dry ashing and the beaker covered with a watch-glass.   Heating at "low" on an electric hot-plate for an hour or so will usually give a clear digest completely free of carbon.   This procedure dissolves all of the ash and renders any silica insoluble.   The digest is then transferred to a 50 cc. conical centrifuge tube and the silica beaker rinsed with hot water acidulated with hydrochloric acid.   The silica and other insoluble material may then be centrifuged out of the sample or the aluminum and iron may be precipitated by the method previously described.   In cases of material high in calcium it is necessary to boil the ash in hydrochloric acid in order to dissolve the calcium sulfate.

This method of ashing has several advantages over the wet ashing method.   Wet ashing requires the use of large quantities of acids and necessitates much more careful watching than does the dry ashing method.   The amount of aluminum in the acids used in ashing 50 gm. of tissue is appreciable.   Another point is the formation of an insoluble precipitate in the acid which cannot be removed from the Kjeldahl flask.   The wet ashing method has been criticized by Underhill and Peterman (7) who say that aluminum will be dissolved from the glassware.   Lundell and Knowles (15) found Pyrex glassware perfectly satisfactory for the acid digestion of non-ferrous metals.

There is a possibility of loss of aluminum in the dry ashing process, but recovery experiments by numerous investigators have shown that if the temperature is not above red heat the loss is slight, if any.

DISCUSSION

In comparing the data of Underhill and Peterman (16) with those of Myers and Morrison (17) one finds that the values obtained for the aluminum content of dog tissue by the alizarin method are

much higher than those found by the aurin method.   Data are
reported in Table VI, which were obtained by direct comparison
of the two methods on aliquot portions of tissue digests.   The
ashing of the tissue was accomplished by the method described by
Underhill and Peterman.   Where possible 50 gm. of material were
ashed.   The ash was dissolved by boiling in dilute sulfuric acid
and diluted to a convenient volume.   The separation of the iron
and aluminum was made on aliquot portions of the digest by the

<div align="center">TABLE VI</div>

<div align="center">*Comparison of Method of Myers, Mull, and Morrison with Method of
Underhill and Peterman on Dry Ashed Tissue*</div>

	Myers, Mull, and Morrison		Underhill and Peterman		Difference
	Amount used	Al per 100 gm.	Amount used	Al per 100 gm.	
	gm.	*mg.*	*gm.*	*mg.*	*mg.*
Beef liver....................	2.0	0.84	0.2	Pptd.	
Dog lung...................	5.0	0.35	0.5	0.80	0.45
" kidney.................	10.0	0.12	1.0	0.48	0.36
" testicle	10.0	0.02	1.0	0.05	0.03
" liver...................	5.0	0.19	0.5	0.75	0.56
" gallbladder bile.........	5.0	0.40	0.5	1.66	1.26
" heart....	10.0	0.18	1.0	0.52	0.34
" intestine...............	10.0	0.33	1.0	0.98	0.65
" stomach................	10.0	0.20	1.0	0.75	0.55
" brain............	5.0	0.37	0.5	1.00	0.63
" bladder................	5.0	0.15	0.5	0.20	0.05
" spleen..................	5.0	0.14	0.5	2.10	1.96
Human blood...............	10.0	0.10	1.0	0.20	0.10

methods described by the authors.   The aurin determinations
were compared in Nessler tubes.

The difference between the "aluminum" as determined by the
two methods is very large.   It would appear that either aluminum
was lost in the manipulations of the Myers, Mull, and Morrison
method or else the alizarin reacted with some substance other than
aluminum.

Data are given in Table VII, which show that added aluminum
is recovered by either method.

In experiments, with thiocyanate, to remove the iron from the

solutions analyzed both methods gave essentially the same differences. When sodium hydroxide was used to remove the iron, low values were found by the alizarin method. These low values have been found to be due to inhibition of color formation due to high salt concentration. The low values obtained by use of aurin

TABLE VII

Recovery of Added Aluminum by Methods of Myers, Mull, and Morrison and Underhill and Peterman

Myers, Mull, and Morrison				Underhill and Peterman			
Amount of tissue used	Al added	Al found	Recovery	Amount of tissue used	Al added	Al found	Recovery
gm.	mg.	mg.	per cent	gm.	mg.	mg.	per cent
10.0	0.000	0.013		1.0	0.0000	0.0046	
10.0	0.005	0.017	80.0	1.0	0.0005	0.0050	80.0
10.0	0.010	0.025	120.0	1.0	0.0010	0.0055	90.0
10.0	0.015	0.030	113.0	1.0	0.0015	0.0060	93.0
10.0	0.020	0.035	110.0	1.0	0.0020	0.0065	95.0

TABLE VIII

Analyses of Tissue Digests Free of Iron, Calcium, Magnesium, and Zinc (Dog Tissue)

Amount of tissue		Al found		Difference
		Aurin	Hydroxy-quinoline	
gm.		mg.	mg.	per cent
10	Liver............................	0.0100	0.0105	+5.0
5	"	0.0048	0.0046	+4.1
20	Blood...........................	0.0040	0.0039	+2.5
10	"	0.0023	0.0020	−13
10	Muscle..........................	0.0030	0.0032	−6.6
5	"	0.0017	No color	
10	Spleen..........................	0.0060	0.0055	−8.3

cannot be explained on the grounds of inhibition of color due to phosphorus, since identical checks have been found with 8-hydroxyquinoline in iron-free tissue digests (Table VIII).

The cupferron method of separating iron from aluminum had proved satisfactory on synthetic mixtures of the metals. The fol-

lowing experiment was performed to compare the two dyes on tissue digests which had been treated in exactly the same manner.

Several tissue digests were mixed to obtain sufficient material for a series of analyses. The iron, aluminum, calcium, and probably some other metals were precipitated by ammonium hydroxide and ammonium acetate. In this precipitation the solution was made definitely alkaline. The precipitate was filtered out and dissolved in hydrochloric acid. The copper and iron were removed

TABLE IX

Comparative Analyses with Alizarin and Aurin

Tissue	Al added	Aurin		Alizarin	
		Al found	Al per gm. tissue	Al found	Al per gm. tissue
gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.0000	+		0.0053	0.0053
2	0.0000	0.0012	0.00060	0.0075	0.0037
3	0.0000	0.0020	0.00066	0.0087	0.0029
4	0.0000	0.0028	0.00070	Cloudy	
5	0.0000	0.0034	0.00069	"	
1	0.0050	0.0058	0.00080	0.0089	0.0039
2	0.0050	0.0070	0.00100	0.0114	0.0032
3	0.0050	0.0078	0.00090	0.0176	0.0042
4	0.0050	0.0082	0.00080	Cloudy	
5	0.0050	0.0088	0.00076	"	
2	0.0000	0.0016	0.00080	0.0072	0.0036
2	0.0025	0.0038	0.00065	0.0097	0.0036
2	0.0050	0.0063	0.00075		
2	0.0075	0.0094	0.00090	0.0149	0.0037
2	0.0100	0.0114	0.00070	0.0178	0.0039
2	0.0150			Cloudy	

by precipitation with cupferron and filtration. Slow evaporation and dry ashing were employed to remove the excess cupferron. The ash was dissolved in hydrochloric acid and made up to volume so that 1 cc. was equivalent to 1 gm. of the tissue. The "aluminum" was estimated by means of aurin and alizarin.

The results are given in Table IX. The variations of aluminum content of iron-free tissue digests, as determined by the two dyes, indicate that either an inhibitory substance was present or that some other material was present that reacts with the alizarin

but not the aurin. The increased color lake with alizarin was likely due to calcium and probably some of the other metals which are not precipitated by cupferron. That the color was not due to iron was shown by negative finding with thioglycolic acid by the method described by Lyons (18).

Further studies were made to see if it might be possible to obtain the same results with aurin and alizarin. Since both of these dyes gave negative results on the supernatant fluid after precipitation of iron and aluminum as the phosphates, it seemed safe to assume that these elements were quantitatively removed from solution. The positive results of the alizarin test on fluids obtained after precipitation of the iron and aluminum in tissue

TABLE X

Comparison of Aurin and Alizarin Estimations of Aluminum in Tissue Digests Free of All Known Interfering Substances

Tissue	Amount used	Al found		Difference
		Aurin	Alizarin	
	gm.	*mg.*	*mg.*	*per cent*
Mixed.....................	5	0.0065	0.0070	7.6
"	10	0.0130	0.0123	5.4
Liver.....................	5	0.0094	0.0100	6.0
"	10	0.0190	0.0190	0
Spleen.....................	10	0.0072	0.0075	4.0
Mixed.....................	10	0.0102	0.0110	8.0

digests seemed to indicate that some other substances were present which reacted with the alizarin. The salt concentration and pH of the solution in both the standard and unknowns must be of the same magnitude if the alizarin method is to be used.

In an attempt to control all of the factors affecting the determination of aluminum by use of alizarin several experiments were carried out in the following manner. The aluminum and iron in tissue digests were precipitated as the phosphates in slightly acid solution. The aluminum and iron precipitates were then dissolved in acid and the iron removed by precipitation with cupferron. In these experiments the precipitate was removed from the filtrate·by filtration through asbestos and kaolin filters. The filtrates were evaporated to dryness and made alkaline with am-

monia and then dry-ashed to remove all of the organic material and the volatile salts.   The residues were dissolved in a measured amount of hydrochloric acid and made up to volume so that 1 cc. of solution was equal to 1 gm. of tissue.   The aluminum was then determined by the aurin and the alizarin methods.   A standard was prepared containing the same amount of aluminum as the sample and also the same amount of hydrochloric acid.   These solutions were always diluted so that there would be about 0.005 mg. of aluminum in 25 cc.   The results of a series of these comparisons are given in Table X.

The data in Table X show that in what might be termed pure solutions of aluminum the two methods agree very closely.   The high values obtained on solutions from which only the iron is removed are probably due to the action of calcium in intensifying the color of the alizarin lake as well as the other elements which form lakes with alizarin.   These data indicate that for routine estimations of aluminum the aurin method is superior to the alizarin method.   A point that favors the use of alizarin is the greater amount of color per unit of aluminum.   The use of the large size colorimeter largely overcomes this advantage, since in this instrument as little as 0.0015 mg. of aluminum can be estimated.

SUMMARY

1. For the determination of aluminum in tissues dry ashing is preferable to wet ashing in view of the large amount of salts in the digest and the difficulty in removing insoluble residues from the digestion flasks.

2. There is always a definitely higher blank when the wet ashing method is employed.   The large amount of sulfuric acid required contains some aluminum.

3. Aluminum and iron can be quantitatively precipitated as the phosphates in order to remove other metallic salts and to concentrate the sample.

4. The separation of aluminum and ferric iron can be made by means of sodium hydroxide, ammonium thiocyanate, or cupferron.

5. Aurin is the most specific and satisfactory reagent for the colorimetric estimation of aluminum.

6. With care in removing all interfering metals and salts identi-

cal results may be obtained by using aurin, alizarin, or 8-hydroxy-quinoline in the colorimetric estimation of aluminum.

7. 1, 2, 5, 8-Hydroxyanthraquinone is a very sensitive reagent for the qualitative testing for aluminum.

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STUDIES ON ALUMINUM

II. THE STORAGE OF INTRAVENOUSLY INJECTED ALUMINUM IN THE DOG

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The intravenous injection of soluble salts of metals with subsequent analysis of excreta and tissues has been a common method of studying the metabolism of metals. The excretion of injected aluminum was investigated by Steel (1) who found that during the 3 days following the injection from 5.55 to 11.11 per cent of the aluminum was excreted in the feces. He did not attempt to determine the fate of the rest of the aluminum. Myers and Morrison (2) found that intravenously injected aluminum was excreted in bile and urine. They also showed that intraparenterally introduced aluminum was stored in the heart, intestine, kidney, liver, abdominal muscle, and skin. Underhill, Peterman, and Steel (3) showed that intravenously injected aluminum was excreted in the bile and urine and through the intestinal wall. The amount excreted did not account for the amount which had disappeared from the blood during the period of investigation. This pointed to storage, but no attempt was made to determine the tissues involved.

EXPERIMENTAL

It seemed desirable to study the storage of injected aluminum by means of tissue analysis. The method employed was the aurin method previously described (4) (Dog 1 was analyzed by the method of Myers, Mull, and Morrison). The methods used in separating the iron and aluminum were the sodium hydroxide, cupferron, and thiocyanate methods; but, since these all were found to agree, the method of separating the aluminum is not specified.

The analyses of five apparently normal dogs are given in Table I. These values agree quite closely with those reported by Myers and Morrison (2).

In a preliminary experiment (Protocol 1) a dog was injected with 1 mg. per kilo of aluminum as $AlCl_3$ in saline.   The results of this experiment agree qualitatively with those of Myers and Morrison (2) and Underhill, Peterman, and Steel (3).

Four dogs were given ten daily intravenous injections of 1 mg. per kilo of soluble aluminum.   At 78, 125, 137, and 150 days, respectively, the dogs were killed and the tissues analyzed.   In

TABLE I

Mg. Aluminum Content per 100 Gm. of Normal Dog Tissue

Tissue	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
Brain	0.37			0.10	0.10
Lungs	0.35	0.13	0.15	0.12	0.09
Heart	0.18	0.09	0.10	0.06	
Liver	0.19	0.18	0.14	0.09	0.11
Gallbladder and bile	0.40		0.21	0.24	
Pancreas		0.04	0.08		
Spleen	0.14	0.06	0.06	0.05	0.08
Stomach	0.20	0.10		0.07	0.02
Intestine	0.33	0.26		0.10	0.10
Blood		0.02	0.02	0.00	0.00
Gluteal muscle		0.05		0.00	
Kidney	0.12	0.08	0.06		0.03
Testicle	0.02				

Table II the initial and final weights of the animals and the results of the analyses of the tissues are given.

DISCUSSION

The average aluminum content of dog tissue found in this study is not significantly different from that reported by Myers and Morrison (2).   No explanation can be given for the high values, particularly of the brain and lungs of Dog 1.   The previous history of the animal is unknown.

In the injection experiment on Dog 6, killed 53 minutes after the injection of 1 mg. of aluminum per kilo of body weight, an imme-

diate distribution of the metal is shown.   Attempts at removal of the aluminum by both the liver and kidney are indicated.   These data agree with the observations of Myers and Morrison (2) and Underhill, Peterman, and Steel (3).   In Table II the analyses of Dogs 7 to 10, killed 78, 125, 137, and 150 days, respectively, after the injection of ten 1 mg. per kilo doses of aluminum, are compared

Protocol 1

Dog 6. Female, weight 7.3 kilos.

9.00 a.m   Catheterized for normal urine and normal blood sample taken.

9.17 "   7.3 mg. of Al as $AlCl_3$ in 50 cc. of saline injected into jugular vein

9.20   Catheter inserted

9.30   Urine flow stopped; 6.5 gm. taken for analysis

9.30   Injected sodium barbital (225 mg. per kilo)

10.00   Ether administered

10.10   Bled to death by cutting the jugular vein.   Immediately dissected and tissues analyzed

The separation of iron and aluminum was made by means of cupferron.

	Al per 100 gm. tissue or fluid
	mg.
Normal urine	0.03
Urine 13 min. after injection	0.18
Bladder urine	1.21
Normal blood	0.00
Blood at death	0.13
Gallbladder and bile	0.48
Lung	0.11
Heart	0.16
Liver	0.25
Spleen	0.19
Pancreas	0.02
Gluteal muscle	0.09
Kidney	0 21

with Dogs 21 and 22 taken from the paper of Myers and Morrison (2) and the normal averages established in this study.   In general it would appear that the aluminum gradually leaves the blood and is stored chiefly in the liver, spleen, and kidney.   The spleen appears to be the last of the three organs to increase in aluminum content.

Intravenously injected aluminum is promptly excreted by way of the urine and bile, as well as stored, so that the amount circulating in the blood is rapidly decreased.

TABLE II

Aluminum Content of Dog Tissue after Injection of Aluminum

Dog No............................	1-5	20*	21*	7	8	9	10
Weight before injection, kg......				15 5	9.0	12.3	9.8
" at death, kg............				19 5	15.5	17 8	12 2
Time after last injection, days..	Controls	8	34	78	125	137	150
Al per kilo injected, mg.........		8 1	6 6	10	10	10	10
Tissue	Mg. Al per 100 gm. tissue						
Blood before injection....				0 00	0.00	0.013	0.00
" at time of death..	0 01	0.18	0 06	0.05		0 02	0.09
Brain....................	0.19					0.12	0.03
Lungs...................	0.17			0.15	0 12	0.18	0.16
Heart...................	0 11	2.00	0.60	0.10	0.04	0.11	0.11
Liver...................	0.14	2 25	0.87	0.83	1.00	1 09	1.96
Gallbladder and bile.....	0.28	0.32	0 34	0.41	0.40	0.27	0.26
Pancreas................	0.06			0.04	0.14		
Spleen..................	0 07			0.26	1 02	1.26	1.56
Stomach................	0.09				0.11	0.00	0.13
Intestine...............	0.19	1.87			0.24	0.20	0 21
Gluteal muscle..........	0.02			0 12	0.18	0.05	0.03
Kidney.................	0.07	1 56	0 33	0.18		0.17	0.11
Urine...................				0.02	0 10	0 01	

* Data taken from Myers and Morrison (2). Aluminum given intraperitoneally.

SUMMARY

1. The aluminum content of normal dog tissue has been determined.

2. The results indicate that the values reported by Underhill and Peterman (5) are too high.

3. Intravenously injected aluminum appears to be widely distributed in the tissue immediately after injection.

4. Injected aluminum is stored primarily in the liver and spleen, but also in the kidney.

5. Injected aluminum promptly appears in the bile and urine.

6. Injected aluminum appears to be retained in the tissue of the dog for long periods.

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THE INFLUENCE OF METHOD OF PREPARATION AND OF CATIONS ON THE ISOELECTRIC POINT OF OVALBUMIN*

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In an extended study of the factors influencing the apparent isoelectric point of ovalbumin, data concerning the effects of a number of buffer anions on this property have been previously reported (13). This work has been continued by observations with varied cations, especially multivalent ions, and by a comparison of the isoelectric point of the protein prepared by different methods.

Methods and Materials

Egg albumin was crystallized from sodium sulfate solutions according to Cannan (5), and from ammonium sulfate solutions according to Sørensen (14), half of the same mixed lot of egg whites being treated by each method. The processes of dialysis, concentration, and preparation of the stock solution were the same as those already described (13).

The various buffer series were prepared from acetic acid and from the acetates of barium, magnesium, and lithium, from the hydroxides of ammonium, sodium, and potassium, from the carbonates of calcium and strontium, and from lanthanum oxide. When the acetates or hydroxides were used, the calculated amount was added as a solution to the required quantity of acetic acid.

In the case of magnesium acetate it was necessary to add 1 per cent of ammonium acetate to the solution to prevent the formation of a colloidal precipitate at pH values greater than 5; this addition was made to all members of this series.   When carbonates or oxides were used in the preparation of the buffers, a weighed quantity of dry material was placed in each flask, and acetic acid was then added.

The methods of observation were identical with those previously reported (13).   0.1 M HCl (pH 1.07) was the standard of reference in the pH estimations, and collodion particles formed the adsorbing substrate in the cataphoresis measurements.   The apparent isoelectric point was determined by graphical interpolation as the pH value corresponding to no electrophoretic migration.   The concentration of protein in all cases was approximately 0.1 per cent.

Three concentration series, each composed of six solutions within the desired pH range, were studied for each cation, and, in addition, sodium acetate buffers were prepared for both sodium sulfate and ammonium sulfate products.

Effect of Crystallizing Salt

Fig. 1 shows the apparent isoelectric points of dialyzed ovalbumin which had been crystallized from ammonium sulfate and from sodium sulfate.   Sodium acetate buffers at three ionic strengths were used for each product.   The differences between the two preparations are small, probably within the experimental error at the lower two ionic strengths and about equal to it at an ionic strength of 0.1.   The point of extrapolation at 0 ionic strength is identical for the two, being pH 4.85.

Effects of Cations

Fig. 2 shows the results obtained with acetate buffers in which the metallic cations were varied.   The solutions used have been described above.   All the pH-ionic strength relations are linear and extrapolate to the same value at 0 ionic strength, pH 4.86 ±0.01.   This differs by only 0.01 pH from that found in the anion series (13).   The slopes of the various lines are roughly proportional to the valences of the cations used, although they also vary for individual ions of the same valence.   The sodium and potassium lines are probably not significantly different.
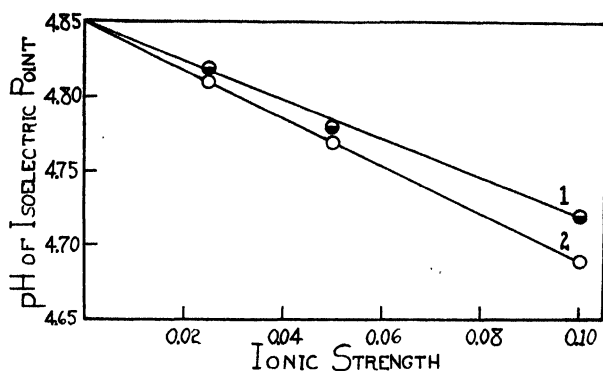
FIG. 1. The effect of the crystallizing salt on the apparent isoelectric point. Curve 1 represents sodium sulfate; Curve 2, ammonium sulfate.
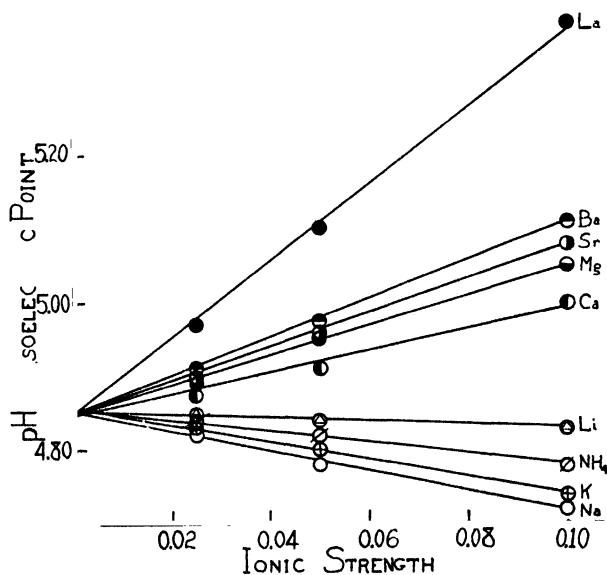
FIG. 2. The effect of various acetate buffers on the apparent isoelectric point. The various lines given on the chart represent the different buffers.

DISCUSSION

It has again been demonstrated that the apparent isoelectric point of ovalbumin is influenced in a definite manner by the nature

and concentration of ions other than hydrogen and hydroxyl in the buffer used. The relations between the ionic strength of the medium and the pH of the isoelectric point are always linear, but the slope of the line varies with the different ions and is, in general, positive for the multivalent cations and negative for the univalent cations, with the anions so far investigated (13). However, the extrapolated values for the isoelectric point in a solution free from ions are identical, within 0.02 pH unit, although each of these values is obtained from measurements with a different added salt. This result gives strong support to the view that the isoelectric point of ovalbumin is at pH 4.86.

In the earlier paper (13) attention was called to the discrepancy between this value and the figures recorded by Abramson (1, 2) and by Tiselius (15, 16). At that time it was thought possible that the use of sodium sulfate instead of ammonium sulfate as the crystallizing salt for the protein might account for a part of the difference. This point has now been tested by the simultaneous preparation of crystalline egg albumin, from the same source, by both methods. The values obtained for the isoelectric points of the two are nearly identical and do not account for the variation between our results and those of the investigators mentioned.

The magnitude of the effect produced by any ion on the apparent isoelectric point is proportional to its valence (see Fig. 2). Multivalent ions cause an apparent rise; Tiselius (16) observed a similar increase in isoelectric point for egg albumin when he replaced sodium with barium in his acetate buffers.

There is a specific effect on the apparent isoelectric point produced by each ion studied; an explanation of these phenomena is difficult to formulate. Three suggestions have been put forward by different investigators, and a fourth may be added. Loeb (7) believed that the valence of any ion determined its action; this is qualitatively true (see Fig. 2), but the differences among ions of a given valence exceed the experimental error and make this explanation inadequate.

Michaelis and Rona (9, 10, 12) studied the influence of salts on the optimal flocculation pH for serum albumin (denatured); they found an ion series, by groups, which, they said, paralleled their charcoal adsorption experiments. They therefore concluded that selective adsorption might account for their results. Michaelis later, however ((8) pp. 144, 145), states that in the case

of true salt formation (which he defines as the formation of a compound, dissociating according to the mass law, between an ion of the ampholyte and some ion of an added salt) the isoelectric point and the pH for maximal flocculation are no longer identical. Andrezjewski (4) investigated the flocculation of gelatin by alcohol. He found that the addition of $MgCl_2$ increased the amount of alcohol needed if the pH were below the isoelectric point; whereas, if the pH were above that point, $MgCl_2$ decreased the amount of alcohol needed. His results would be predicated from the present study on the basis of a shift in the isoelectric point produced by the $MgCl_2$. Tiselius' barium effects have already been mentioned.

A third hypothesis has been stated by Ljalikov and coworkers (6), who recently presented work on the changes produced by electrolytes in the isoelectric point of gelatin. They found a diminution proportional to the concentration with various sodium and potassium salts, whereas multivalent ions produced rises comparable in magnitude to those we have observed. These investigators explain their results as hydration and valence effects.

A fourth possible explanation of the specific ion effect may be postulated from the definition of the isoelectric point of a simple ampholyte, which may be formulated thus:

$$\overset{+}{A}\overset{+}{H_2} - \overset{+}{H} \rightleftharpoons \overset{-}{A}\overset{+}{H} \rightleftharpoons \overset{-}{A} + \overset{+}{H} \tag{1}$$
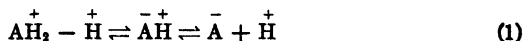
$$K_1 = \frac{(H)(AH)}{(A\overset{+}{H_2})} \qquad\qquad K_2 = \frac{(H)(A)}{(\overset{-}{A}\overset{+}{H})} \tag{2, 3}$$

$$K_1 K_2 = \frac{(\overset{+}{H})^2(\overset{-}{A})}{(A\overset{+}{H_2})} \tag{4}$$

At the isoelectric point

$$[A\overset{+}{H_2}] = [\overset{-}{A}] \tag{5}$$

Hence

$$(\overset{+}{H})^2 = K_1 K_2 \cdot \frac{\gamma A\overset{+}{H_2}}{\gamma \overset{-}{A}} \tag{6}$$

$$\text{pH (isoelectric point)} = \tfrac{1}{2}(pK_1 - pK_2) - \tfrac{1}{2}\log \frac{\gamma A\overset{+}{H_2}}{\gamma \overset{-}{A}}$$

Parentheses refer to the activity and brackets to the concentrations. γ is the activity coefficient of an ion. K_1 and K_2 are true thermodynamic constants and do not change. Therefore pH (isoelectric point) varies with the logarithm of the activity coefficient ratio, and such a logarithm has been found to be proportional to the ionic strength (3). Hence, pH (isoelectric point) would be a linear function of the ionic strength, which we have found to be the case. A similar specific ion influence has been observed with other properties; for example, in the ionization constant of lactic acid (11).

SUMMARY

The nature of the crystallizing salt used in the preparation of egg albumin shows very little or no effect on the isoelectric point of the protein.

The pH of the isoelectric point is linearly related to the ionic strength.

The influence of each cation is roughly proportional to its valence, but each ion has a specific effect.

Extrapolation to 0 ionic strength of the apparent values for the isoelectric point of ovalbumin, cataphoretically determined, in acetate buffers of a number of cations, gives an isoelectric point of pH 4.86 ± 0.02.

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ON THE MECHANISM OF LYSOZYME ACTION*

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Lysozyme, the purification and chemical properties of which have recently been described (1), was considered by Fleming (2) to be an enzyme. Its heat stability rather suggested a physicochemical action. Furthermore, some substances, such as rattlesnake venom, saponin, digitonin, cholic, choleic, and desoxycholic acids, have been found to bring about appreciable, but incomplete, lysis of organisms sensitive to lysozyme.[1] This may be explained by the finding that these organisms themselves contain lysozyme which is released by the above substances.[2] Moreover, a very potent, highly purified lysozyme preparation produced no appreciable lowering of surface tension in water or saline, whereas a dilute solution of caprylic alcohol, which had a surface tension about 60 per cent that of water, had no lytic effect.

With suitable substrates no protease, kinase, amylase, lipase, or phosphatase activity could be demonstrated. On the other hand, the action of lysozyme upon *Sarcinæ* brings about progressive increase in non-protein nitrogen, inorganic phosphorus, and reducing substances.

We have been able to confirm the suggestion of Hallauer (4), that lysozyme acts on a mucoid fraction of the *Sarcinæ*. We believe that this action explains its bacteriolytic property and that

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[1] The organisms used were very resistant to solution either by autolysis or by the action of alkali.

[2] According to Avery and Cullen (3), autolysis is prevented and the lytic action of bile salts on pneumococcus is abolished, if an autolysin, present in the bacteria, is inactivated.

the increase in non-protein nitrogen and inorganic phosphorus is due to the release of material enclosed by the bacterial membrane.

From our results it appears that the action is on one of the sugar linkages in certain mucoids. The nature of this linkage, which is certainly not α-glucosidic, is unknown. The action is quite specific, for a number of glucoproteins and polysaccharides are not susceptible. The maximum liberation of reducing groups is, with the *Sarcinæ* fractions, about one-third of the total reducing power after acid hydrolysis. It is conceivable that the first action of the lysozyme on the *Sarcinæ* consists in the breakdown of the aggregates of the mucoid micellæ in a manner comparable with the first step of the action of diastase on starch (5).

In view of the relative abundance of lysozyme in egg white, experiments were carried out with egg mucoids and polysaccharides as substrates. It was found that these products were attacked in the same way as the bacterial fractions only when the use of alkaline hydrolysis in their preparation had been avoided. Similarly, bacterial fractions lost their susceptibility after more drastic alkaline hydrolysis. The idea that this may be due to the removal of acetyl groups is supported by the observation that the treatment of one of the poorer bacterial substrates with acetic anhydride in pyridine doubled the amount of reducing substances liberated by the same lysozyme preparation. This is significant in the light of the recent work of Avery and Goebel (6) on the rôle of the acetyl groups in the immunological behavior of pneumococcus polysaccharides and of the observation of Helferich, Iloff, and Streeck (7) that, while emulsin is without action upon phenol-β, d-glucosaminide, the N-acetyl derivative is readily split.

In general, mucoids are resistant to the action of proteolytic enzymes (8). The inhibitory effect of egg white preparations on tryptic activity (9, 10) is probably due to a mucoid. A sample of highly active antitrypsin, kindly sent us by Dr. Swenson, showed the properties and composition of an egg mucoid (without lysozyme activity), and some of our egg mucoid preparations, tested by Dr. Swenson, had antitryptic activity.

Avery and Cullen (3) have obtained an enzyme from various types of pneumococcus, which renders the organism Gram-negative. In higher concentrations, the ferment causes lysis of the bacterial membrane. In recent experiments, as yet unpublished,

Dubos (11) has shown that our lysozyme preparations have the same action. Although these enzymes are much alike in their chemical properties, they are probably not identical.

It is probable that enzyme-substrate pairs similar to this from egg white exist elsewhere. One such ferment was found in a commercial pepsin preparation which liberated reducing sugar from gastric mucin. This ferment was not identical with pepsin, by which it was destroyed. It may be identical with Castle's ferment (12).

<div align="center">EXPERIMENTAL</div>

Preparation of Lysozyme from Sarcinæ—Sarcinæ, grown on large agar plates, were carefully washed with saline and distilled water, and dried with acetone and ether. The organisms were ground for

<div align="center">TABLE I</div>

Effect of Lysozyme on Non-Protein Nitrogen of Sarcina Suspension

	Total N	Non-protein N per cc.		
		0 hr.	2 hrs.	24 hrs.
	micrograms	*micrograms*	*micrograms*	*micrograms*
Lysozyme + saline................	614	548	544	544
Bacteria + "	956	13	14	20
" + lysozyme............	1570	560	637	912

12 hours with saline in a ball mill, during which treatment the larger part of the organisms remained intact. The mixture was centrifuged, acidified, again centrifuged, and the supernatant solution was precipitated with flavianic acid. The precipitate was dissolved in saline to make a 0.45 per cent solution which gave upon assay complete lysis up to a dilution of 1:64. This preparation was also active towards another lysozyme-sensitive organism (*Sarcina lutea*). Like lysozyme from egg white, it was inactive towards *Escherichia coli communis*. By use of the same procedure no lysozyme was obtained from the non-susceptible *Escherichia coli*.

Chemical Observations during Lysis—Table I illustrates the increase in non-protein nitrogen of a mixture of lysozyme and bacteria killed by heating at 80° for 30 minutes. . Aliquots of the

incubation mixtures were precipitated with a quarter volume of 20 per cent trichloroacetic acid, and nitrogen was estimated in the filtrate by the micro-Kjeldahl method. Similar results were obtained with fresh or acetone-dried bacteria.

To test the possibility of lysozyme being a protease, it was incubated with casein, peptone, and a mixture of proteins prepared from the sensitive *Sarcinæ* as substrates. No increase in carboxyl groups (13) could be observed. No phosphatase activity of lysozyme could be demonstrated, with use as substrates of magnesium hexosediphosphate, sodium β-glycerophosphate, lecithin, and a lipid mixture from the *Sarcinæ*. Tests for lipase activity were negative, and fluoride, which inhibits lipase, did not inhibit lysozyme activity.

For the study of the increase in reducing material, substrates were prepared from the *Sarcinæ* and from egg white. Folin-Wu (14) or Hagedorn-Jensen (15) sugar analyses were made on mixtures of equal volumes of (usually) 1 per cent substrate and 0.01 to 0.1 per cent lysozyme solutions incubated at 37°. A drop or two of toluene sufficed to avoid contamination.

Experiments with Bacterial Substrates—The bacterial substrates were alkaline hydrolysates of washed, acetone-dried organisms, fractionated by alcohol precipitation, containing 4 to 9 per cent nitrogen, depending upon the alkalinity and time of hydrolysis.

Acetone-dried *Sarcinæ* were extracted successively with weak alkali, weak acid, and 90 per cent acetic acid. The residue was digested with 2.5 N NaOH at 100° for 10 to 30 minutes; the supernatant liquid was brought to 50 per cent alcohol; the precipitate was redissolved in water and brought to 75 per cent alcohol. A few drops of glacial acetic acid were added to promote precipitation. The precipitate was again dissolved in water and reprecipitated with 75 per cent alcohol and acetic acid, washed with acetone, and dried. The products formed slimy solutions which gave a very strong Molisch reaction, but gave no color with iodine and reduced Benedict's solution only after acid hydrolysis.

One such solution contained 0.479 per cent dry material which had 0.72 per cent ash (0.95 per cent of the ash was phosphorus). The nitrogen content on an ash-free basis was 6.33 per cent (atomic ratio of N:P, 15.3). When incubated 40 hours with a

lysozyme preparation (acid 50 per cent acetone extract[3] of egg white powder, containing both lysozyme and mucoid[4]), the reducing sugar (Folin-Wu) of the mixture increased 126 micrograms per cc.

Table II represents a similar but more detailed experiment. Substrate A-404 contained 6.73 per cent nitrogen and 19.5 per cent reducing sugar as glucose after hydrolysis. Substrate A-

TABLE II

Effect of Lysozyme on Reducing Power of Bacterial Sugar Complex

Equal volumes of substrate (1 per cent) or saline, lysozyme (1 per cent) or saline, and M/15 phosphate (pH 6 or 7). Hagedorn-Jensen sugar method after $Zn(OH)_2$ precipitation.

	Total sugar per cc. mixture after acid hydrolysis	Per cent of total substrate sugar liberated					
		With phosphate, pH 6			With phosphate, pH 7		
		0 hr.	21 hrs.	64 hrs.	0 hr.	21 hrs.	64 hrs.
	micrograms						
Lysozyme + saline.................	1250						
Substrate A-404 + saline..........	975						
" A-405 + "	1200						
" A-404 + lysozyme........	2150	4.6	23.3	27.4	3.2	16.7	24.4
" A-404 + " *.......	2150		17.7	25.9			
" A-405 + "	2250	7.8	22.3	32.1	7.1	16.3	25.4

* Without toluene.

405 contained 5.88 per cent nitrogen and 24.0 per cent reducing sugar.

[3] An early procedure for preparation of lysozyme involved the extraction of 4 gm. lots of egg white powder overnight in the cold with 200 cc. of 50 per cent aqueous acetone containing 1 per cent HCl. The supernatant solution was then brought to 80 per cent acetone and a small amount of concentrated ammonia was added (to introduce salt) to facilitate flocculation. After standing in the refrigerator 24 hours, the precipitate was centrifuged off and washed with acetone and ether. The 350 to 400 mg. of dry white powder obtained, consisting chiefly of one of the egg mucoids, was easily soluble and had a lytic activity of 13 to 51 units per mg. This procedure was abandoned because of the cost of materials used.

[4] In all preparations containing mixtures of mucoid and lysozyme high blank reductions were usually obtained, undoubtedly due to action of the enzyme upon the mucoid before the experimental period began.

With highly purified lysozyme solutions in corresponding dilutions similar results were obtained.

Experiments with Egg White Substrates—A crude polysaccharide obtained by baryta hydrolysis[5] gave no increase of reducing material after incubation with lysozyme. A similar but more pure material prepared according to Levene and Mori (16) gave the same negative results despite many variations in the experimental conditions. In the procedure of Levene and Mori the hydrolysis is carried out with 10 per cent $Ba(OH)_2$ for 8 hours. We found that, if the egg white was heated for only 10 minutes with 2.5 N NaOH, the resulting sugar complex was resistant to lysozyme.

TABLE III

Effect of Lysozyme on Reducing Power of Egg Mucoid

Equal volumes of substrate (1 per cent egg mucoid Preparation 80-B) or saline and lysozyme or saline. Hagedorn-Jensen sugar method without $Zn(OH)_2$ precipitation.

	Sugar per cc. mixture	
	0 hr.	21 hrs.
	micrograms	*micrograms*
Lysozyme + saline............................	0	15
Mucoid + saline.............................	221	225
" + lysozyme..........................	270	395

With 5 minute alkaline hydrolysis positive results were obtained, but even then the products gave only a small increase in reducing substances with the enzyme. However, when alkaline hydrolysis was avoided and a mucoid was prepared from acid aqueous or acid alcoholic extracts of the acetone-dried egg white powder, increases in reducing sugar comparable to those with bacterial materials were obtained.

Egg white, diluted four times and acidified with acetic acid, was poured into boiling water. The supernatant fluid was concentrated under reduced pressure and neutralized to maximal precipitation. The precipitate was extracted with 0.1 per cent HCl, and the extract treated with flavianic acid. The precipitated

[5] We thank Dr. P. A. Levene for this material.

flavianates (containing most of the lysozyme) were removed and the clear supernatant solution poured into 6 volumes of alcohol. The results with this preparation (No. 80-B) are shown in Table III. It will be noticed that this material had a high reducing blank.[4] It was found to contain lysozyme when tested with *Sarcinæ*. The non-protein nitrogen of the mixture increased 109 micrograms per cc. during the 21 hours.

The egg mucoid prepared by acetic acid-alcohol extraction (1) was likewise acted upon by lysozyme. A greater activity was observed in acid solution than in neutral. *Sarcinæ* swell markedly in acidic medium in the presence of lysozyme, but are not lyzed; upon neutralization they immediately dissolve, even after removal of the lysozyme (4, 17).

We have found no action by lysozyme on starch,[6] glycogen,[6] cartilage, gastric mucin, the polysaccharide acid from the vitreous humor (18), osseomucoid, chitin, or a mucoid prepared from *Escherichia coli communis*.

SUMMARY

The lytic action of lysozyme on susceptible bacteria has been studied. Lysis cannot be explained on a physical basis; *e.g.*, lowering of surface tension. Lysozyme has no protease, kinase, amylase, lipase, or phosphatase activity. It liberates reducing sugar from mucoids or polysaccharides of the susceptible *Sarcinæ* and from a mucoid fraction of egg white. The type of linkage attacked is not known.

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[6] Controls with dilute filtered saliva showed a marked increase in reducing power.

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CHEMISTRY OF SLASH-PINE (PINUS CARIBÆA, MORELET)

II. FATS, WAXES, AND RESINS OF THE GROWING TIPS

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The flow of oleoresin from the trunk of the slash-pine is maintained and stimulated by periodic wounding. Certain interesting phenomena ensue, which suggest a significant relationship between the process of formation of new tissue and oleoresin production. Within a few days after wounding, the white phloem in the immediate vicinity of the wound, especially at the sides, develops a green pigmentation which is presumably chlorophyll. This green color in the phloem has been observed normally only in the tissue of the last 3 years' apical growth of the tree and, especially in the last year's growth, is associated with large vertical resin passages in the phloem itself, which are filled with resin. No resin passages occur in the corresponding year's growth of phloem below the tip region. Further, the newly developing tips of the slash-pine, which comprise the current year's growth, are highly chlorophyllous and resinous. In cross-section the young phloem contains numerous organized vertical resin passages.

With the appearance of pigmented phloem, around the wound, the development of callous tissue begins and continues for years, until the wound is healed. The suggestion arises that resin formation is a property of certain types of parenchymatous tissue and that wounding greatly stimulates the formation of this parenchyma in the vicinity of the wound, thus causing a condition analogous to that in the highly parenchymatous growing tips in which abundant resin formation is a normal function.

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* Maintained at Madison, in cooperation with the University of Wisconsin.

Free resin acids were not found in the phloem (1), but this material was comparatively rich in fats, tannins, starch, and sugars.   Since the fats of the phloem are well characterized, it became of interest to examine the fats, resins, and waxes of a tissue rich in resin and also highly parenchymatous.   The young growing tips are such a material.

Consequently, an examination of the petroleum-soluble fraction (60–70°) of the dried tips has been carried out.   All crystalline preparations were dried for analysis at 56° and 0.1 mm. for 24 hours over phosphorus pentoxide.   Rotations were observed with a Franz Schmidt and Haensch (Berlin) quartz wedge saccharimeter equipped with a Ventzke scale and an electric sodium lamp as light source.   The melting points were made with a Thiele apparatus with a thermometer calibrated against a Bureau of Standards thermometer and are not corrected for stem emergence.   Carbon and hydrogen were determined by a semi-micromethod developed from the original Pregl procedure.

EXPERIMENTAL

The material used in this investigation was collected from slash-pine saplings in the forests near Cogdell, Georgia, from April 5 to 12, 1934.   At this time the growing tips were in process of elongation and were from 3 to 12 inches long.   Needle development had not yet begun and very little wood had been formed.   The tips were collected in the morning and prepared for drying in the afternoon.   Since they could not be dried in a reasonable time while whole, it was necessary to shred them longitudinally with a pocket knife and dry the subdivided tips in a current of air at 45°.   Under these conditions they were reduced to a brittle condition in about 18 hours.   The unshredded tips required 4 to 5 days for even incomplete drying.

The dried tips were coarsely broken up, packed in a galvanized drum, and shipped to Madison.   They were then ground on a Wiley mill to approximately 60 mesh and the ground material was placed in storage at 3° in air-tight containers.   About 20 kilos of material were thus prepared.

A preliminary examination by means of selective extraction of a small quantity in a Soxhlet apparatus gave the following results.

Solvent	Per cent extracted
Petroleum (60–70°)...................................	4.9
Ether...	4.5
Ethyl acetate.......................................	6.6
Alcohol..	13.0

For purposes of a complete examination of the petroleum extract, the available material, roughly divided into three portions, was exhausted with a petroleum fraction (60–70°) in a Lloyd extractor. The extracts were combined and the solvent removed mostly at atmospheric pressure and finally under slightly reduced pressure. The resulting extract was a very deep green semi-resinous mass.

Volatile Products—The whole extract was subjected to distillation with steam and by this process about 50 cc. of colorless oil with a slight terpene odor were recovered. By shaking out the oil with sodium bisulfite solution in the usual way, a trace of oil with aldehydic characteristics was obtained, but it was too small for identification. From the aqueous portion of the distillate and by extraction of the oil with sodium carbonate solution a very small quantity of an acid was recovered, which had the odor of caprylic acid but from which no pure derivative could be obtained.

The remaining oil, free from aldehydes and acids, was fractionally distilled through an 8 inch Widmer column followed by a 6 inch Vigreux column with the results shown in Table I.

The first three fractions absorbed no bromine and behaved as saturated paraffins. The method of their preparation (the use of a petroleum fraction) prevents any conclusions as to their existence in the tissue. At least 25 cc. of the distillate consisted of saturated hydrocarbons boiling well above the solvent and there is thus raised the interesting question in biogenetics of the existence in these tips of paraffins analogous to the *n*-heptane of the oleoresins of *Pinus jeffreyi* and *Pinus sabiniana*. The question will be answered, it is hoped, by the steam distillation of a large quantity of fresh tips.

From Fraction 4, a nitrosochloride was prepared by the method of Wallach (2). Only a few mg. of crystals were obtained, which melted at 106–107° with decomposition. This gave a nitrolpiperidide melting at 116–117°. These properties, combined with

the physical properties and odor of the impure fraction, constitute a fairly good identification of α-pinene.

Non-Volatile Products—The extract after steam distillation was taken up in hot alcohol. Upon cooling, a bulky flocculent precipitate was separated by filtration (Fraction A). The solution after the separation of Fraction A was saponified with alcoholic potassium hydroxide and the greater part of the alcohol removed under a vacuum. After dilution with water, the unsaponifiable matter (Fraction B) was removed by continuous extraction with ether. When the fatty and resinous acids (Fraction C) were liberated from their potassium salts in the presence of ether by means of dilute sulfuric acid, an insoluble substance (Fraction D) was deposited at the water-ether interface. This was removed by centrifuging.

TABLE I

Distillation of Volatile Oil

Fraction No.	Amount	B. p.	n_D^{25}	Remarks
	cc.	*°C.*		
1	6.2	77– 81	1.405	Widmer column
2	19.5	81–110	1.4105	" "
3	6.0	110–150	1.419	Vigreux "
4	7.8	150–168	1.461	" "
5	7.2		1.4875	Residue

Examination of Fraction A

Isolation of Melissic Acid—The greenish, waxy precipitate, Fraction A, obtained as described above, was dissolved in isopropyl ether and the solution was decolorized by evaporation with activated charcoal and subsequent solution in alcohol. The fractions obtained by evaporation of this solution were non-uniform, so the whole was saponified with alcoholic potassium hydroxide, containing a little benzene. Since the resulting potassium salt was only slightly soluble in cold dilute alcohol, difficulty was encountered in the subsequent ether extraction for removal of the unsaponifiable material. Therefore, the saponification mixture was evaporated to dryness and the residue digested several times with hot isopropyl ether. The material so removed was examined with Fraction B. The residue insoluble in isopropyl ether was taken up in hot water, and the wax acid liberated with dilute sulfuric acid

and crystallized as platelets from a 75:25 mixture of isopropyl ether and ethyl acetate to a constant melting point of 90–91°. The determination of the neutralization equivalent in alcohol was extremely difficult because of the great insolubility of the potassium salt.

Analysis
$C_{30}H_{60}O_2$. Found.   C 79.20, H 13.48, neutralization equivalent 120.5
          Calculated.   " 79.64, " 13.27,   "   "   123.8

The *p*-bromophenacyl ester melted at 98–99°.

Although no good criterion of purity is available, this substance is certainly a high wax acid and most probably melissic, present partly free and partly as esters.

Examination of Unsaponifiable Matter (Fraction B)

The main portion of the unsaponifiable material was recovered from its ethereal solution and dissolved in hot alcohol. The first fractions deposited upon cooling melted at 133–135° and gave sterol reactions. A portion was purified by means of the digitonin procedure (1) and the regenerated sterol found to have a constant melting point of 138–139° with no depression, when mixed with the sitosterol previously obtained from the phloem (1).

Analysis—$[\alpha]_D^{25} = -26.26°$
          $C_{29}H_{50}O$. Found.   C 83.98, H 12.61
                  Calculated.   " 84.06, " 12.08

The acetate melted at 126°. The sterol was therefore a sitosterol and identical with that previously isolated from the phloem.

Isolation of Melissyl Alcohol and n-Nonacosan-10-ol

The later fractions of unsaponifiable material from the solution from which the sterol had been isolated were combined with the unsaponifiable portion from Fraction A. From this material traces of sterol were removed by means of digitonin. The purified material was then fractionally crystallized from alcohol and fractions obtained melting from 79–83°. The various fractions were acetylated independently and the acetates fractionally crystallized from isopropyl ether. By combining corresponding fractions

and refractionating, there were obtained a less soluble acetate melting at 73–73.5° and a more soluble one melting at 44–45°.

The acetate melting at 73–73.5° was saponified according to the technique of Vesterberg and Vesterberg (3).

Saponification equivalents of 116.34, 115.4, average 115.87, were obtained, which corresponded well to that required for the acetate of an alcohol $C_{30}H_{62}O$, 116.89. The regenerated alcohol crystallized in mossy form, melted at 84–85°, and solidified at 83°.

Analysis

> $C_{30}H_{62}O$.   Found.   C 81.96, H 14.07
>   Calculated.   " 82.19, " 14.15

This alcohol is definitely a saturated C_{30} alcohol, corresponding well in its properties with those of the melissyl alcohol of carnauba wax, the melting point of which is variously reported with maximum values of 88°, and which gives an acetate, m. p. 74°.

The acetate melting at 44–45° was saponified as above, and saponification equivalents of 120.06, 119.95, 119.5, average 119.87, were obtained. $C_{31}H_{62}O_2$ requires 120.4. The regenerated alcohol crystallized in needles and melted sharply at 83–83.7°. When the material was mixed with the melissyl alcohol described above, the melting point was depressed 7°.

The properties of this alcohol agree well with those of the *n*-nonacosan-10-ol described by Chibnall (4), who gave a melting point of 81.9–82.2° and an acetate melting point of 44.5–45°, and by Kawamura (5).

In order further to establish its identity, it was converted according to the directions of Kawamura (5) to the corresponding ketone by oxidation with potassium dichromate in glacial acetic acid. The ketone melted at 74.5–75.5°. Chibnall gives 74.7–74.9° and Kawamura gives 74–75° for the melting point of *n*-nonacosan-10-one.

The oxime of the above ketone was also prepared according to Kawamura (5). Crystallized from alcohol in fine long needles, it melted at 51–51.7°. Kawamura reported 49–50°.

Professor Chibnall kindly examined a specimen of this alcohol and reported a melting point of 81.7–82° for it alone and mixed with his specimen obtained from apple wax. He also supplied us with specimens of the ketone and its oxime, the melting points and

mixed melting points of which agreed with the above values. There is no doubt as to the identity of the alcohol as *n*-nonacosan-10-ol.

The oily tailings were very complex and could not be resolved into individual compounds.

Examination of Fraction C

Fatty and Resinous Acids—This fraction was extremely difficult to separate into its constituents, and yields of the fatty acids were very small because the bulk of the material was a complex mixture of resin acids and green pigments.

The crude mixture was subjected to the lead salt-alcohol method of Twitchell (6). By this means a partial separation of solid acids, contaminated with resin acids and liquid acids, was effected. The soluble lead salts were further purified through their solubility in ether, when a further portion of lead salts was recovered from which the resin acid discussed below was prepared.

Solid Acids—The insoluble lead salts were freed from lead and 75 gm. of the mixed resin and fatty acids esterified in methyl alcohol with dry HCl according to Twitchell's procedure (7). During the process a very large proportion of resin acids separated. After evaporation of the supernatant solution, the residue was taken up in petroleum (60–70°) and the solution repeatedly shaken out with dilute potassium hydroxide. Upon removal of the solvent, about 3 gm. of green esters remained. This was dissolved in isopropyl ether, digested with activated charcoal, and evaporated to dryness. The residue gave a pale yellow solution in alcohol from which colorless leaflets deposited upon cooling. After several crystallizations these reached a constant melting point of 61° and showed no depression when mixed with the methyl behenate previously obtained from the phloem.

The mother liquors, from which the small amount of methyl behenate was obtained, were saponified with alcoholic potassium hydroxide and the insoluble potassium salt formed was separated. From this an acid was recovered in very small yield, melting at 77° and giving a neutralization equivalent of 165. Behenic acid melts at 78–79° and gives the same neutralization equivalent. This acid was therefore behenic acid.

The acids were recovered from the soluble potassium salts from

the above saponification and repeatedly crystallized from alcohol. The total yield of about 0.2 gm. melted at 59–60° and gave a neutralization equivalent of 212. (Palmitic acid, m. p. 62.5°, neutralization equivalent 219.) The p-bromophenacyl ester was prepared and melted at 84°, solidifying at 76°. (Palmitate, m. p. 86.5°, solidifying point 79°.) Mixed with the known palmitate, it melted at 86° and solidified at 78°. This was, therefore, palmitic acid.

Liquid Acids—The ether-soluble lead salts obtained as described above were decomposed in the usual manner and the mixture of resin acids and liquid fatty acids esterified according to Twitchell's procedure (7). There were obtained about 4.5 gm. of crude esters from which a very dark oily mixture of acids was obtained by saponification. The acids were converted to the potassium salts and the aqueous solution of these repeatedly extracted with ether. By this process a considerable proportion of neutral substances was removed. The acids were recovered from the purified potassium salts and found to weigh 0.5 gm. The neutralization equivalent was 195.6, indicating C_{18} acids. The acids were again recovered and the iodine value determined as 145.6. This value indicates a mixture of oleic and linoleic acids, such as was found in the bark.

Resin Acids—In the processes of separating the very small amounts of fatty acids, the resin acids had undergone considerable violence and, for the most part, were discarded. However, a fraction of lead salt, soluble in alcohol, but insoluble in ether, had been obtained, which, having endured little treatment of violent character, was investigated. The acids recovered in the usual manner gave the usual color reactions of the resin acids of the abietic type, but were heavily contaminated with green pigments, and uncharacterizable substances. The crude mixture was dissolved in ether and the solution shaken out with ammonium carbonate solution. The acid recovered from this extract was dried *in vacuo* and distilled under a vacuum of < 0.005 mm. with the oil bath at 250–260°. The distillate was light yellow and consisted of a solid which separated under a layer of heavy oil. The latter was removed by rinsing with acetone, and the solid fraction crystallized from acetone. After three crystallizations from alcohol containing a trace of hydroquinone, the acid melted con-

stantly at 158.5–159°.   It was colorless and beautifully crystalline in triangular prisms characteristic of abietic acid.   It gave a neutralization equivalent of 185.9 ($C_{20}H_{30}O_2$ requires 185.4), $[\alpha]_D^{25} = -4.85°$ (4 per cent in alcohol).   The melting point corresponds to that of the abietic acid of Ruzicka (8), 158°, but the rotation differs (Ruzicka, $-68°$).   This is probably one of the large number of abietic acids.

The other resin acid fractions were too badly contaminated and metamorphosed to warrant examination.

Fraction D.   Isolation of a Sterolin

When the potassium salts from the original saponification mixture were decomposed in the presence of ether, a precipitate appeared at the water-ether interface.   This was separated by centrifuging, washed with ether, and repeatedly crystallized from dioxane.   It melted constantly at 227° and gave the Liebermann-Burchard reaction for sterols, and corresponded, therefore, to a similar impure preparation obtained from the phloem.

It was hydrolyzed by refluxing for 40 minutes in a mixture of 30 cc. of amyl alcohol, 10 cc. of 15 per cent hydrochloric acid, and sufficient alcohol to make a homogeneous solution (9).   The alcohols were removed by distillation with steam and the sterol taken up in ether.   Recrystallized from alcohol, it gave a constant melting point of 138° and gave no depression when mixed with the free sterol obtained before.

The water-soluble portion of the hydrolysate was neutralized and a phenylosazone prepared, which melted at 204–205°.

The substance was therefore a sterolin of the sterol which occurs in the free state.

SUMMARY

A petroleum ether (60–70°) extract of the young growing tips of Pinus caribæa, Morelet, yielded the following substances: paraffins of the C_5–C_9 range, a trace of α-pinene, melissic acid, melissyl alcohol, n-nonacosan-10-ol, a sitosterol and a sitosterolin, palmitic and behenic acids, oily acids apparently consisting of oleic and linoleic acids, and abietic acid.

All combustions and rotations were performed in the Bio-chemical Research Laboratory of the University of Wisconsin by Dr. Eugene Schoeffel. The services of this Laboratory were placed at our disposal by Professor Karl Paul Link.

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GRAVIMETRIC METHODS FOR THE DETERMINATION OF TOTAL BODY PROTEIN AND ORGAN PROTEIN*

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Although much has been learned about protein metabolism by such indirect methods as N_2 balance experiments, there are still elementary questions to which no answer can be given until direct measurements of the protein content of the various organs and tissues of the body have been made. With the exception of methods for protein in plasma (1) and muscle (2) the gage of the protein content of the body has hitherto been taken as given by its nitrogen content. But even if all non-protein nitrogen could be removed, it is evident that this is not a satisfactory procedure for estimating the sum of a mixture of proteins whose nitrogen concentration is known to vary from 10 per cent to 18 per cent. On this account and in order to investigate certain problems which require a direct approach and precise results the following gravimetric methods have been devised. They have been gradually developed during the past 5 years and in their present form have been shown to be reliable and accurate.

The general procedure is first to prevent autolysis and to render the proteins insoluble by immediate immersion of the carcass and excised organs in a boiling 0.5 M sodium acetate-acetic acid buffer solution kept at pH 5. The material is then reduced to a form in which it can be sampled by methods which vary with the physical character of each organ or part. Water-soluble substances are washed out with acetate buffer, fats and acetate with hot alcohol, and finally water is removed by drying *in vacuo*. The remainder is weighed. Non-protein material which cannot be removed, such as minerals derived from bone, and glycogen

that cannot completely be washed out from the liver are determined and subtracted.   The material thus measured is regarded as giving the nearest approximation to the amount of the protein structure required for the manifestation of vital phenomena, the essential machinery stripped of storage material and accessories and separated from the medium within which it operates.

Sampling of the material can be avoided whenever the mass is not too great.   In our work we were anxious to eliminate, as far as possible, the factor of individual variation, so we have used a highly standardized strain of albino rat and have made each measurement on the mixed organs and carcasses of thirty animals. They were males between the ages of 90 and 110 days and under ordinary conditions their carcasses weighed about 5000 gm., their livers 250 gm., and their kidneys about 45 gm.   These quantities were all too large to be conveniently handled without sampling. In the case of the hearts, however, which did not weigh much more than 20 gm., and of course in all smaller organs it was possible to deal with the whole material without sampling.

Liver Proteins—It is not necessary to reduce the livers to a fine powder before the material is sampled, because in this case complete homogeneity can be attained by converting them into a form which corresponds to a *pâté de foies gras*.   Each boiled liver is ground in a mortar with enough buffer solution to make a thick soup and all of them are transferred to the bowl of a household utensil known as a "Kitchen Aid."   This is a motor-driven apparatus which keeps a fenestrate paddle revolving through the soup. As the stirred brei thickens it becomes a stiff paste and finally attains a putty-like consistency.   In that state it gathers together into a self-adherent ball which can be transferred without any loss in handling.   The whole mass is then quickly divided into parts of convenient size, placed in air-tight stoppered vessels, and weighed on an analytical balance.

The samples taken varied from 2 to 6 gm. in weight, corresponding to from 2.5 to 7.5 gm. of fresh liver.   They were transferred from the weighing bottles to 50 cc. centrifuge tubes, and ground in about 35 cc. of 0.5 M sodium acetate solution of pH 5 by means of a stout rod.   There was nothing left now of any cellular structure.   Under the microscope only a suspension of particles could be seen.   This suspension was left overnight at 0°

and was centrifuged the next morning.   The opalescent supernatant fluid on which some fat floated was decanted through a Whatman seamless fat extraction thimble.   The process of grinding in buffer solution, centrifuging, and decanting was repeated four times.   The liver paste was then washed with 95 per cent alcohol from the centrifuge tube into the thimble which was plugged with cotton-wool.   This thimble and the plug of cotton-wool had previously been extracted with hot alcohol, dried, and weighed in a weighing bottle.   After being washed with absolute alcohol, the material in the thimble covered by the cotton-wool was extracted in a Soxhlet apparatus for 18 hours with absolute alcohol.   No measurable amount of material was removed by longer extraction or by the use of other fat solvents.   The thimble containing the protein was then returned to its weighing bottle and the alcohol removed by evaporation in an oven at 110°. There still remained the difficulty of drying protein to a constant weight.   This was only overcome after we had attached a water-cooled oil pump to a vacuum oven and were able to maintain an almost complete vacuum at a temperature of 80° for 48 hours. Under these conditions further drying has no measurable effect and the protein may be weighed.

In the case of the liver we did not succeed in completely washing out all glycogen.   Even when the grinding and suspension in buffer solution were repeated eight times the supernatant fluid still contained a little glycogen, and so it had to be measured and subtracted from the protein-glycogen weight.   The dry protein takes up water with great rapidity and the transfer of part of it to another weighing bottle must be done as quickly as possible and either in a box filled with dry air or within an oven.   Quantities of from 0.5 to 1 gm. were taken for analysis.   The method of Sahyun was used (3).   In fasting experiments and in a series in which thyroxine was given no measurable quantity of glycogen was found in the washed protein.

This liver protein method has a high degree of accuracy.   In the livers from each group of thirty rats eight protein determinations were always made and the mean deviation from the average was 0.3 per cent.   A similar order of accuracy was reached for the protein in other organs.

Kidney Proteins—The capsules of the kidneys were removed and

they were opened and blotted with filter paper before they were weighed and coagulated. The sampling is most conveniently accomplished by grinding the boiled kidneys with a little buffer solution in a large mortar until the material adheres together in a putty-like mass which can be subdivided, sampled, washed, extracted, and dried as in the case of the liver. Only traces of glycogen too small to measure were found in the dried protein and as no variation under widely different dietary conditions was observed, we have not found it necessary to carry out glycogen determinations as a routine procedure.

Heart Proteins—The heart was cut out always by the same individual, using the same technique. The apex was held by forceps and the heart pulled up out of the pericardial sac, while the inferior vena cava and pulmonary vessels were divided by scissors. The aorta and superior vena cava were cut just above the still beating auricle. Both ventricles and auricles were opened and any adherent blood removed with cotton-wool before the heart was weighed and boiled. Then all the thirty hearts were ground together in a mortar and separated into four parts which were weighed and transferred to four centrifuge tubes for washing. The total heart protein was then determined in the same way as for the liver and kidney.

Proteins of Gastrointestinal Tract and Other Organs[1]—In most of our work the spleen, adrenals, pancreas, bladder, seminal vesicles, and testicles along with the abdominal and pelvic fat pads were removed *en mass* with the gastrointestinal tract and all these organs and tissues were boiled together in buffer solution. The whole tract was then pulled out into a straight line, opened with a razor blade, and the contents washed out under running water from a tap. After drying overnight on glass plates in a current of air at a temperature of 64°, the whole mass was collected in the bowl of the Kitchen Aid and stirred with acetone, transferred to a Buchner funnel, the acetone filtered off, the material returned to the Kitchen Aid, stirred with ether, and the

[1] When the brain and testicles are treated separately, a small amount of protein passes through the thimble with the acetate buffer. This protein is retained on refiltering after the filtrate has been heated with 3 times its volume of alcohol.

ether filtered off. After this treatment it was found that everything could be reduced to a fine gray powder by grinding in a mortar. The whole powder was allowed to become air-dry, weighed, and the samples treated in the same way as in the case of the liver.

Blood Proteins—All the blood which could be obtained from the opened abdominal aorta while the heart was still beating was collected. It was centrifuged at high speed and the serum drawn off and weighed. Acetate buffer (5 times the volume of serum) was added and the protein was coagulated by raising the temperature to the boiling point, while the mixture was being stirred. The precipitate was filtered off on a Buchner funnel and washed with hot absolute alcohol. The material then contracted into a sponge-like mass which could be sampled without loss. The method thereafter was the same as that used for the liver, kidney, and heart. The clot was weighed and boiled in buffer solution which was later filtered off under pressure. It was then washed with acetone, transferred to a mortar, ground to a powder, allowed to become air-dry, weighed, sampled, and treated as were the organs.

Carcass Proteins—The carcass is the exsanguinated rat with the heart excised, stripped of all abdominal and pelvic organs, and of the removable abdominal and pelvic fat. Each of the thirty carcasses was boiled in buffer solution and then chilled by storage in a room at $-2°$. While still cold they are minced in a meat chopper. If they are ground while warm some of the "gelatin" may be lost. The mince is spread on glass plates and dried at $64°$ in a current of air for 24 hours, and is thus reduced to about 40 per cent of its original weight. The material is then stirred with about 3 times its weight of acetone. The acetone is filtered off and the remainder is stirred with ether and filtered again. The material is now ready for grinding to a fine powder in a Wiley mill. The ether must be removed by suction. If it is too dry there may be a slight loss by the escape of a very fine powder during the powdering process and it may then be necessary to sprinkle it with a little acetone. In the mill the powder is passed through a fine mesh screen. It ordinarily weighs about 1500 gm. It is collected in an open can and when it has become air-dry it is weighed on a Sartorious balance sensitive to 0.1 gm. It is not convenient to

take samples of more than a few gm. in weight and it is evident that in material consisting of such diverse constituents as hair, bone, connective tissue, and muscle one would not be surprised to find considerable errors in sampling.   At first we attempted to attain thorough mixing by shaking the powder.   The average discrepancy in the first eleven experiments between determinations on the same materials was 0.7 per cent.   But it was later observed, when we were dealing with very emaciated rats in which the fur was a relatively greater part of the whole, that this shaking led to a separation and balling together of the hair particles, and in these experiments the average error in one case rose to as high as 2.9 per cent.   Since then shaking has been given up and the powder from the mill has been layered over a large area after being passed through a fine screen.   Even so the sampling is not very accurate and we have felt it best to wash a minimum of eight samples from each lot of carcasses.   The samples are placed in centrifuge tubes, suspended in buffer solution, and washed in the same way as are the organ proteins.   The supernatant fluid contains some hair particles but these are not lost since the fluid is decanted through a fat extraction thimble.   After Soxhlet extraction with hot alcohol, to remove the remaining traces of fat, and drying *in vacuo* at 80° the protein and the bone minerals are weighed.   The extraction with the acetate buffer solution at pH 5 had liberated CO_2 from the calcium carbonate of the bone, but it is not necessary to try to define the exact nature of the mineral residue, for the total weight can be determined by dry ashing.   For this purpose the thimble containing the protein and minerals is placed in a double porcelain crucible as recommended by Stolte (4) and heated over a flame until the major part of the carbon has been oxidized. It is then transferred to a cold muffle furnace and the temperature raised over the next 3 hours to about 770° before it is cooled and weighed.   The weight of minerals subtracted from the protein-mineral weight of the sample gives the protein content.   Up to the time when this method was devised the protein content of the carcass was determined indirectly from the N_2 content of the washed and alcohol-extracted samples.

The preparation of the carcass powder for sampling is a somewhat tedious and time-consuming procedure.   It is none the less necessary if measurements of the distribution of protein in the

body under widely varying conditions are to be compared. It would for instance be of no avail to obtain an accurate measure of the protein in the liver in well fed rats as compared with the protein content of the liver of fasted rats if we compared these quantities in terms of, say, 100 gm. of body weight. Casual inspection is sufficient to show that 100 gm. of the well fed animals containing a relatively large proportion of fat is far from being the equivalent of 100 gm. of the lean fasted rats. A valid comparison requires that these organ quantities be expressed as gm. per 100 gm. of the total body protein of the individuals from which the organs were derived, and, of course, apart from any consideration of organs or parts, the determination of the protein of the whole

TABLE I

Protein Distribution in Thirty Rats Reared on Adequate Diet

	Fresh weight per rat	Protein weight per rat	Protein distribution, gm. per 100 gm. total protein
	gm.	*gm.*	
Carcass..........................	194.70	35.82	85.9
Liver............................	8.98	1.79	4.3
Kidney..........................	1.48	0.26	0.6
Heart...........................	0.83	0.14	0.3
Remainder.......................	44.22	3.72	8.9
Total.........................	250.21	41.73	100

body is an essential instrument for a direct attack on many important problems in protein physiology.

The nitrogen concentration in protein preparations under varying experimental conditions averages 16.02 per cent for the liver protein, 15.91 per cent for kidney protein, 16.20 per cent for heart protein, and 16.60 per cent for carcass protein. We had hoped that we might be able to wash out most of the minerals after boiling the organs at a pH of 5 in acetate buffer, but the ash content was only reduced to about 30 per cent of the amount present in the unwashed liver, to 23 per cent in the kidney, and to 18 per cent of the original amount in the case of the heart. The ash content of the dry protein preparations was accordingly high, 2.2 per cent for liver, 2.0 per cent for kidney, and 1.3 per cent for heart protein.

Table I gives the protein distribution in a group of thirty rats, between the ages of 90 and 110 days, reared on an adequate diet.

SUMMARY

1. Gravimetric methods for the measurement of the total protein of the body or of any of the organs of the body are described.

2. The total protein content and the protein content of various organs of well fed rats arc given.

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DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

V. THE DESATURATION OF FATTY ACIDS IN THE ORGANISM*

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About 25 years ago Leathes and Wedell (1) proposed the well known theory, that desaturation was the first step in the breakdown of fatty acids. Many investigators have dealt with the problem, but no definite proof that desaturation occurs in the living organism has ever been given. Experiments in which the desaturation of fatty acids has been deduced from the increase of the iodine numbers are inconclusive, inasmuch as the organism can also synthesize unsaturated fatty acids from non-fatty material. The observed increase in the iodine numbers in such experiments can with equal probability be due to a synthesis of unsaturated fatty acids.[1] Equally inconclusive are experiments in which the presence of unsaturated fatty acids has been demonstrated in the lymph after feeding saturated acids. These results could be explained by a transportation of unsaturated acids with lymph or blood from other organs to the intestinal system.

The experiments here reported demonstrate that unsaturated fatty acids containing deuterium can be isolated from the fat of mice which have been fed saturated fatty acids containing deuterium. This observation definitely proves desaturation.

EXPERIMENTAL

Preparation of Saturated Fatty Acids—30 gm. of the methyl esters of the fatty acids from linseed oil dissolved in 30 cc. of di-

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[1] For a review of recent work on enzymatic desaturation of fatty acids by Italian authors see (2).

butyl ether were shaken with platinum oxide in an atmosphere of deuterium until complete saturation had occurred (3).   The crystalline product was saponified with potassium hydroxide in methyl alcohol and the fatty acids were recrystallized from dilute alcohol.

Analysis of the water obtained from the combustion of the acids showed that the deuterium content of the fatty acids was 11.2 atoms per cent (4).   The iodine number according to the method of Rosenmund and Kuhnhenn (5) was 0.0.

The above fat was fed to two groups of mice.

Feeding Experiment I—Nine mice were fed for 7 days with bread in which 10 per cent of the saturated fatty acids and 0.15 per cent of cholic acid had been incorporated, the latter being added to facilitate absorption.   The mice took the food well. The fatty acids of the total animals (8.57 gm.) were isolated according to the method already described (3).   The iodine number was 86.7 and the atom per cent of deuterium was 1.35.   A sample of the body fluid was distilled from the carcasses before they were worked up for fat.   This water contained 0.23 atom per cent of deuterium.

Feeding Experiment II—Three mice were fed 12 days with the same diet as in Experiment I.   The deuterium content of the saturated fatty acids in the diet was 8.66 atoms per cent.   4.46 gm. of fatty acids were isolated.   The atom per cent of deuterium was 1.7 and the concentration of deuterium in the body fluids was 0.21 atom per cent.

Separation of Unsaturated Fatty Acids—In view of the high sensitivity of our analytical method, contamination of the unsaturated acids with saturated acids would lead to incorrect results. It was therefore essential to separate all of the deuterium-containing saturated fatty acids from the unsaturated material.   In general, there are two types of methods available for the separation of saturated acids (6): (1) The saturated acids are precipitated from a mixture of fatty acids as insoluble metal salts.   (2) They are separated by distillation of the brominated methyl esters. The saturated bromine-free esters distil over first due to their much lower boiling points.

None of the methods of the first type is exact; appreciable amounts of saturated fatty acids always remain in the mother liquor.   The methods of the second type give much better results.

We did not use them, however, because at the high temperature necessary for distillation the brominated fatty acids tend to lose hydrogen bromide. This breakdown might lead to a loss of deuterium.

For our purposes we have altered the method of Twitchell (7) for the precipitation of fatty acids with lead acetate in such a way that we were able to separate the last traces of deuterium-containing saturated acids from the unsaturated acids.

We used deuterium as an indicator to study the completeness of such precipitations. The principle resembles that underlying the use of radioelements as indicators (8). When a mixture of saturated acids containing deuterium and unsaturated deuterium-free fatty acids is treated with an excess of lead acetate, about 5 to 10 per cent of the saturated acids remains in solution. On

TABLE I

Fractionation of Fatty Acids

Fraction	Iodine No.	Deuterium content
		atoms per cent
Precipitate A.............................	4.9	8.0
" B.............................	14.4	0.27
" C.............................	9.7	0.00
Fraction D, unsaturated acids............	98.8	0.00

now adding to the mother liquor saturated fatty acids of natural origin, the new precipitate contains the bulk of the deuterium-containing saturated fatty acids which remained in solution. By repeating this procedure, the last detectable traces of deuterium can be removed from the mother liquor.

For example, 1 gm. of the same deuterium-rich saturated fatty acids as were fed to the mice and 3.5 gm. of a mixture of unsaturated fatty acids of natural origin (iodine number, 98.8) were dissolved in 45 cc. of 95 per cent alcohol and precipitated hot with 4 gm. of lead acetate in 40 cc. of alcohol. After 24 hours the crystalline precipitate (A) was filtered off. A mixture of 0.5 gm. of stearic acid and 0.5 gm. of palmitic acid in alcohol was added to the mother liquor. After filtering off the newly formed precipitate (B), the procedure was repeated (Precipitate C). The precipitates and the final mother liquor (Fraction D), which contained

the lead salts of the unsaturated acids, were decomposed with HCl and ether according to the usual method. The analysis of the fatty acids thus obtained is given in Table I.

With this method it is possible to "wash out" the last traces of the saturated fatty acids originally present. An appreciable amount of the unsaturated fatty acids is carried down in the several precipitations, as is shown by the iodine numbers of Precipitates B and C.[2] This loss is of no importance for our experiments.

Isolation of Unsaturated Fatty Acids from Mice—The total fatty acids from the mice were dissolved in 10 parts of hot 95 per cent alcohol and treated with an equal volume of a 10 per cent

TABLE II

Isolation of Unsaturated Fatty Acids from Mice Fed Saturated Acids Containing Deuterium

Fraction	Experiment I			Experiment II		
	Amount	Iodine No.	Deuterium content	Amount	Iodine No.	Deuterium content
	gm.		*atoms per cent*	*gm.*		*atoms per cent*
Starting material..............	7.3	86.7	1 35	4.46		1.7
Precipitate A...................	2.6	3.3	2.60	1.61	20.6	2.58
" B................		9.8	0.30		10.9	0.25
" C................		10.5	0.07		11.0	0.15
Fraction D, unsaturated acids..	3.8	125.6	0.48	2.03	74.7	1.16

alcoholic lead acetate solution. After 24 hours the precipitate (A), containing the lead salts of the saturated fatty acids, was filtered off and washed with alcohol. To the mother liquor was added twice a mixture of 0.5 gm. of stearic acid and 0.5 gm. of palmitic acid (Precipitates B and C). All precipitates thus obtained and the mother liquor (Fraction D), which contained the lead salts of the unsaturated acids, were decomposed as before. The analyses of the fatty acids are given in Table II. During the process of fractionation approximately 15 per cent of the fatty acids was lost.

[2] The first precipitate also contains some saturated acids which were present in the unsaturated oil, and accounts for the low deuterium content of this fraction.

The continued precipitation (B and C) did not yield deuterium-free products as in the preliminary experiments, for small amounts of deuterium-containing unsaturated fatty acids were carried down each time. The iodine number and the deuterium content in Precipitate C run more or less parallel and indicate a 10 per cent content of deuterium-containing unsaturated fatty acids from Fraction D.

There is no doubt that the high deuterium content of Fraction D is due only to the unsaturated fatty acids. The large difference in the deuterium content of Fractions D in the two experiments may be due to the longer duration of Experiment II.

DISCUSSION

The results obtained permit no other interpretation than that the unsaturated fatty acids were derived *directly* from the saturated ones which were fed.

In our experiments the body fluids contained about 0.2 atom per cent of deuterium which came from the breakdown of the ingested fatty acids. Since the deuterium content of the unsaturated fatty acids (0.48 and 1.16 atoms per cent) was from 2 to 5 times as much as the body fluids, it is impossible that the deuterium in these acids came from the water.

During the process of desaturation a certain amount of deuterium may be taken from the molecule. We cannot, therefore, use the values for quantitative calculations. We hope, however, to gain further insight into the process by varying the experimental procedure and by isolating individual unsaturated fatty acids.

SUMMARY

Mice were fed saturated fatty acids containing deuterium; the fatty acids of the entire animals were freed of the last traces of deuterium-containing saturated fatty acids by a method developed for this special purpose. The high deuterium content of the unsaturated fatty acids so secured proves conclusively that fatty acids can readily be desaturated in the organism.

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STANDARD IODINE SOLUTIONS

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Dilute iodine is being used with increasing frequency in biochemical methods. Its standardization and stability are therefore matters of importance.

When 0.1 N iodine in 0.12 M KI is diluted with distilled water and titrated immediately without further addition of KI, there is a loss in equivalence as shown in Table I.

That this is an apparent loss in iodine only and not a true loss due to volatilization at the time of preparing the dilution is shown in the following way.

A small quantity of iodine is dissolved in water without any potassium iodide. This solution is then used to titrate a dilute solution of arsenious acid (0.0001 N), with starch as the indicator. At the end-point KI is added to make about a 5 per cent concentration (0.3 M). The solution then becomes an intense blue. The additional amount of iodine made available for titration by the addition of the potassium iodide is now determined in a second titration with 0.0001 N arsenious acid. The sum of the amounts recovered in the two titrations is now assumed to be the total amount of iodine in solution. With use of this value several dilutions in distilled water are made as indicated in the first column of Table II. Each of these solutions is then titrated in the same way as that described above for the original solution. It is quite apparent that the use of KI makes these dilute solutions much more nearly equivalent.

The fact that one is titrating to the permanent disappearance of a slowly disappearing color leads to an overtitration and partly explains the recovery of more than 100 per cent in the second titration in the last two instances.

TABLE I

Loss in Equivalence of Iodine Solutions

Calculated iodine concentration	Factor with As_2O_3	Percentage difference
N		
1 (10^{-1})	$\times$ 0.989	0.0
2 (10^{-3})	$\times$ 0.940	5.0
2 (10^{-4})	$\times$ 0.852	13.9
4 (10^{-5})	$\times$ 0.534	46.0
1 (10^{-5})	$\times$ 0.325	67.2

TABLE II

Effect of KI on the Titration Error of Dilute Iodine Solutions

Iodine in distilled water, calculated	First titration equivalence with As_2O_3 (0.0001 N)	Second titration after addition of KI to 0.3 M	Per cent recovery	
			First titration	Second titration
N	N	N		
0.000580	0.000508	0.000073	87.6	12.6
0.000290	0.000203	0.000087	70	30
0.000145	0.000080	0.000081	55	56
0.000058	None	0.000061	0	105

TABLE III

Equivalence and Stability of Dilute Solutions of Iodine in 0.3 M KI

Date	Solution	I_2 concentration in 0.3 M KI	Factor with As_2O_3	Remarks
1935		N		
Mar. 26	A	0.1	1.120	
Apr. 12	"	0.1	1.123	
Mar. 26	B	0.002	1.114	Solution B, diluted from Solution A
May 6	"	0.002	1.113	on Mar. 26, no KI in receiving
Oct. 2	"	0.002	1.105	flask; loss 0.8% in 6 mos.
" 2	C	0.002	1.123	Diluted from Solution A, 0.3 M KI in receiving flask
Mar. 26	D	0.050	1.220	
Nov. 11	E	0.001	1.220	Diluted from Solution D, 0.3 M KI final concentration

Hubbard (1917) used 0.18 M KI to make his dilute iodine more permanent. Clausen (1922) used 0.12 M KI. Although these concentrations decrease the titration error and do increase the stability of the solutions, I have found neither of them adequate. With 0.3 M KI the dilute solutions are exactly equivalent with arsenious acid in the same dilution and are stable over several months. In addition there must be a final concentration in the titration of 0.3 M KI. Table III illustrates some of these points.

The stability of these dilute solutions of iodine in 0.3 M KI, as shown in Table III, indicates that both KI and I·KI³ compounds are stable in the presence of free iodine in these concentrations.

It is important that the potassium iodide be tested before use for free iodine, since commercial samples of potassium iodide may show some free iodine. As is very well known also, KI solutions very rapidly change on standing, liberating free iodine, especially in light. It is essential, therefore, that the KI solution be made up just before use.

The color of the starch-iodine compound with an excess of iodine is red rather than blue (Mylius, 1895). The color change at the end-point is somewhat slowed with the high concentration of KI but not sufficiently to make the titration impractical. The first permanent tinge of color is taken as the end-point.

Our standard arsenious acid was repeatedly checked and did not change during the period of its use.

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THE TOTAL ELECTROLYTE CONTENT OF ANIMALS AND ITS PROBABLE RELATION TO THE DISTRIBUTION OF BODY WATER*

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Although the knowledge of the total amounts as well as the composition of both extracellular and intracellular fluids of the body is necessary for understanding the metabolism of water and electrolyte, at present accurate information is limited to the concentrations of electrolytes in extracellular fluids. Since previous work (1–3) has shown that the concentrations of electrolytes in various extracellular fluids are fairly closely predicted from the concentrations in venous serum, the latter may be used to measure the concentrations in all extracellular fluids. It follows that the volume of extracellular water could be computed from the concentration of a given ion if the total amount of that ion present in extracellular fluid were known.

In the present paper these concepts are applied to analyses of whole animal bodies and organs, together with analyses of venous serum. The data permit the calculation of the total amounts of both extracellular and intracellular water, as well as the concentrations of electrolytes in these fluids.

Methods

Preparation of Tissues—After removal of as much blood as possible by vein or cardiac puncture the animals were sacrificed. The hair was cut off with clippers and the contents of the gastrointestinal tract were removed and discarded after weighing. The

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animals were then skinned, dismembered, and ground in a meat chopper. The difference between the weight of the animal before skinning and the combined weight of the ground material was considered to be water lost by evaporation during the manipulations.

Four animals were analyzed as a whole without separation of the tissues. In three experiments skin, muscle, liver, skeleton, and viscera were treated separately. The skin included subcutaneous tissue as well as epidermis and the hair which could not be removed with clippers. All the skeletal muscle was separated except the small amount which could not be readily removed without stripping the periosteum. The skeleton included all bone and cartilage, the spinal cord, and a small amount of muscle and tendon attached to the bones. The viscera consisted of all the remaining organs except the liver.

The ground material was weighed and then evaporated to apparent dryness on a steam bath and finally to constant weight in an oven at 105° to obtain the water content. The dried residue was then ground to a fine powder and stored in air-tight bottles for further analyses. In the first experiment nitrogen determinations on aliquots of the fresh ground tissue agreed with the values found after drying, so that in all subsequent analyses aliquots of the dried material were used. In Dogs 1 and 2 and Monkey 1, the dried tissue could not be conveniently handled without first removing part of the fat by extraction with petroleum ether. The extracted lipid was weighed. No electrolyte was lost by this process because of the selectivity of petroleum ether as a fat solvent.

About 10 cc. of the blood removed were immediately transferred to a stoppered tube containing a small amount of mercury. The tube was completely filled, so that when stoppered all air was excluded. The blood was then defibrinated by gentle inversion for about 5 minutes. The remainder of the blood was delivered under mineral oil, allowed to clot, centrifuged, and the serum removed. All analyses were carried out on both defibrinated blood and serum. The volume of the blood cells was determined by centrifuging the defibrinated blood at high speed for 40 minutes in capillary tubes. The water content of defibrinated blood and serum was determined by drying the samples at 105°.

Chemical Methods—Nitrogen was determined by the Kjeldahl method (4); total fat by extraction with alcohol and ether; chloride by the method of Van Slyke and Sendroy (4), with a preliminary alkaline digestion of the tissues as suggested by Sunderman and Williams (5); sodium by the method of Barber and Kolthoff (4) after removal of potassium as described by Hald (6); potassium by Hald's modification of the method of Shohl and Bennett (6); phosphorus by the method of MacKay and Butler (4); calcium by the method of McCrudden (4); magnesium by precipitation as ammonium magnesium phosphate in the calcium-free filtrate and determination of the phosphorus in the precipitate by the method of Fiske and Subbarow (7). For the sodium, potassium, calcium, phosphorus, and magnesium determinations the material was ashed in silicon dishes in a muffle furnace at 500°.

Calculations—The calculations to be described are based on the assumption that all the body chloride may be considered as confined to the extracellular water.[1] The justification for this assumption will be apparent later. The accuracy of the resulting conclusions will depend on the correctness of this major premise, as well as upon the accuracy of the methods employed.

The concentrations of sodium, potassium, and chloride in the extracellular fluid were estimated from their concentrations in serum water by means of the Donnan factor (1, 2), which in all cases is so near 0.96 that this value was used.

Assuming that all body chloride is extracellular it follows that: (1) Total extracellular water equals total chloride divided by the concentration of chloride in extracellular water. (2) Intracellular water equals the difference between total water and extracellular water. (3) Extracellular sodium equals the product of the volume of extracellular water and the concentration of sodium in extracellular water. (4) Extracellular potassium equals the product of the volume of the extracellular water and the concentration of

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[1] The erythrocytes are known to contain about 80 milli-equivalents of chloride per liter of cellular water. This erythrocytic chloride may comprise about 3 to 4 per cent of the total chloride of the body. In the experiments involving the analysis of the separate tissues this source of error is still further reduced, since 30 to 40 per cent of the blood of the animals was removed prior to preparation of the tissues. The data as given do not include a correction for this intracellular chloride, since it was not found to alter the results significantly.

potassium in extracellular water. (5) Intracellular potassium equals the difference between total potassium and extracellular potassium. (6) The concentration of potassium in intracellular water equals the intracellular potassium divided by the volume of intracellular water. (7) The sodium not present in extracellular water equals the difference between total sodium and extracellular sodium. (This has been labeled "excess sodium.")

The concentrations of magnesium and phosphorus in intracellular water were determined by procedures similar to (4), (5), and (6).

The concentrations of sodium and potassium in the red cells were estimated from their concentrations in serum and whole blood by the equation, $C = (B - S(1 - V))/V$ where C represents concentration in the red cell, B, concentration in whole blood, S, concentration in serum, and V, proportion of cells in whole blood.

In the above equations water is expressed in liters; sodium, potassium, magnesium, and chloride in milli-equivalents, and the concentrations of these ions in milli-equivalents per liter of water.

Results

To conserve space the data obtained from the analyses of tissues will not be presented in detail. In Tables I to III, only the results of the application to these data of the calculations indicated in the above equations are given.

Table I represents a summary of the results, describing the distribution of fat, water, and electrolyte in the bodies of seven animals. These represent analyses of the entire body, or the sum of the analyses of the separated tissues. Columns 1, 2, 3, and 4 show the proportion of body weight (exclusive of the hair and gastrointestinal contents) contributed by fat, total water, extracellular water, and intracellular water. The total body water varied between 55 and 75 per cent of the body weight and was roughly inversely proportional to the amount of body fat. On the other hand, the proportion of extracellular water agreed fairly closely in all the animals, ranging from 24 to 30 per cent and averaging 27 per cent of the body weight. The intracellular water, representing the difference between total water and extracellular water, was necessarily dependent upon the magnitude of

the total body water and varied from 29 to 50 per cent of the body weight. In Columns 6 and 7 are given the concentrations of sodium plus potassium in the extracellular water and in the water of the red cell. The calculated concentration of potassium in the intracellular water is given in Column 8. As will be shown later, little or no sodium can be present in the cells of the body if all of the chloride is extracellular. Potassium, therefore, represents practically all of the univalent base of tissue cells. The values in Column 9 represent the expected total sodium in the

TABLE I

Distribution of Water and Electrolyte in Body

Animal		Weight[*]	Per cent of body weight[*] as				Electrolyte in body water, m.-eq. per liter water				Na, m.-eq.		
			Fat	Total water	Extracellular water	Intracellular water	Extracellular Cl	Extracellular Na + K	Red cell Na + K	Tissue cell K	In extracellular water (calculated)	In body	Excess Na
			(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
		kg.											
Rabbit	1	2.37	2.3	73	28	45	116	153	138	149	97	117	+20
"	2	2.02	3.0	75	25	50	124	150	133	135	73	96	+23
"	3	1.83	2.3	75	28	47	107	149		118	72	89	+17
Dog	1	5.81	25.6	55	26	29	123	159	145	148	226	314	+88
"	2	6.27	14.6	64	28	36	126	151	144	141	262	351	+89
Monkey	1	4.04	11.0	65	24	41	113	153		145	144	201	+57
"	2	2.87	2.0	72	30	42	121	157	159	153	128	178	+50

[*] Weight, excluding hair and contents of gastrointestinal tract.

extracellular fluid as calculated by equation (3). In Column 10 is shown the total body sodium as determined by analysis. The difference between these two values, termed "excess sodium," is given in Column 11. The finding of this large amount of excess sodium was at first disconcerting in view of the close agreement which had already been noted between the concentrations of the univalent cations of extracellular water and the potassium of intracellular water. However, to anticipate Table II, the excess sodium was not found in appreciable quantities in any tissue except the skeleton.

TABLE II

Distribution of Water and Electrolyte in Tissues of Dog, Monkey, and Rabbit

Tissue	Weight	Fat (1)	Total water (2)	Extracellular water (3)	Intracellular water (4)	K (5)	Mg (6)	P (7)	Protein (N × 6.25) (8)	Total Na in extracellular water (calculated) (9)	Total tissue Na (10)	Excess Na (11)	Na + K in total water (12)
	gm.					m.-eq.	m.-eq.	mM	gm.	m.-eq.	m.-eq.	m.-eq.	m.-eq. per l.
Dog 2													
Skin........	893	25	55	46	9	232	56	235	2020†	61.9	60.8	−1.1	162
Muscle.....	2448	14	68	17	51	132	30	103	317	62.7	74.6	+11.9	144
Liver......	255	4	73	36	37	129	36	198	491	13.5	13.5	0	140
Skeleton...	1756	13	58	25	33	146			548	63.7	135.8	+72.1	218
Viscera....	710	17	69	38	31	174	38	182	443	39.6	37.5	−2.1	164
R.b.c.......						144*		34	469				144
Monkey 2													
Skin........	252	1.5	70	59	11	229	63	220	2630†	22.2	18.4	−3 8	146
Muscle.....	1116	0.9	79	16	63	143	30	94	314	26.7	29.4	+2.7	149
Liver......	85		79	38	41	132	33	184	395	4.8	5.3	+0.5	151
Skeleton...	1010	3.3	61	30	31	151			702	45.8	97.4	+51.6	237
Viscera....	318	3.3	80	39	41	182	38	183	355	18.4	19.6	+1.2	174
R.b.c......						159*			441				159
Rabbit 3													
Skin.......	189	1.6	71	45	26	120			1160†	12.3	12.3	0	139
Muscle.....	734	1.2	80	16	64	122	36	96	282	17.3	17.0	−0.3	127
Liver......	71		75	31	44	109		166	420	3.2	4.0	+0.8	140
Skeleton...	544	4.5	67	28	39	114			422	22.3	38.7	+16.4	175
Viscera....	220		80	37	43	119	38		284	11.6	12.7	+1.1	139
R.b.c.......									445‡				

* Concentration of Na + K in red blood cell water.

† These obviously high values are due to epidermis and hair.

‡ Because of loss of blood sample red cell determinations were not available in this experiment. The value given is the average of determinations in five rabbits.

In Table II are presented the data pertaining to the distribution of fat, water, sodium, potassium, and magnesium in the individual tissues of Dog 2, Monkey 2, and Rabbit 3. Columns 1, 2, 3, and 4 show the proportion of the tissue weight contributed by fat, total water, extracellular water, and intracellular water. The various tissues of the same animal differ widely in the proportions of extracellular and intracellular water. However, in the analogous tissues of the different animals there is a close agreement in the proportion of extracellular water. In the three animals the extracellular fluid in muscle averages 16 per cent of tissue weight, in skin 50 per cent, in skeleton 28 per cent, in liver 35 per cent, and in the other viscera 38 per cent. In none of the animals are any results far from the averages.

In Columns 5 and 6 of Table II are given the concentrations of potassium and magnesium in the intracellular water of the different tissues. The obviously too great concentration of potassium in the skin of Dog 2 and Monkey 2 may be due to the inclusion of dried epidermal cells and hair which may contain potassium. It is also true that the greatest error in the calculation of cellular concentration would be found here because of the relatively small amount of cellular water in skin. The concentration of potassium in the intracellular water of the viscera of the two animals is also considerably higher than in the other tissues. This is perhaps due to the incomplete removal of the intestinal contents which may be high in potassium. In the remaining tissues, although the values for intracellular potassium show considerable scattering, the concentrations are, nevertheless, of the same magnitude as the concentration of univalent cations in the water of the red cell (Table I). Whether the variations are truly significant or merely represent errors produced by the methods of calculation cannot be stated at the present time. The values for the concentration of magnesium in intracellular water, except for the skin, show a fair agreement in the various tissues, being approximately 35 milli-equivalents per liter.

Columns 9, 10, and 11 show the distribution of sodium in the tissues. The "excess sodium" is given in Column 11. In the case of Monkey 2 and Rabbit 3 practically all of the excess sodium is found in the skeletal system. In the other tissues there is virtual agreement between the amount of sodium found and

the sodium in the extracellular water as calculated.   In the dog about 85 per cent of the excess sodium is found in the skeleton but an appreciable amount is also found in the muscle.   The excess sodium present in the skeleton represents approximately 50 per cent of the total skeletal sodium and about 25 per cent of the sodium in the whole animal.

The concentration of sodium plus potassium in the total water of the tissues is given in Column 12.   Except for the skeleton there is reasonably close agreement between the various tissues of the different animals.   The values in the case of the skeleton, however, are greater by about 35 to 60 milli-equivalents per liter of water.   The significance of this apparently greater concentration of the univalent cations in the water of the skeleton will be brought out later when the nature of the excess sodium is discussed.

In Column 7 is given the concentration of phosphorus in the intracellular water and in Column 8 the concentration of tissue protein in terms of intracellular water.   The corresponding values for the red blood cell are included for comparison.   All of the tissues show a much higher concentration of phosphorus than do the red cells.   The amount of phosphorus found in the muscle cell is, however, only about half as great as that present in other tissue cells.   The concentration of protein in the muscle cells in all three animals is considerably less than that found in the red cell, the muscle cells containing about 300 gm. of protein per liter of intracellular water, whereas the red cells contain about 450 gm. per liter of cellular water.   The protein content of the other tissues of the body tends to resemble more closely that of the red cells.

<div align="center">

DISCUSSION

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The data present the distribution of body water and the concentration of electrolyte in tissue cells as predicted from the assumption that all body chloride except that of the red blood cell is found in extracellular fluid.[2]   The validity of this assump-

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[1] Hanke and Donovan (8) have suggested that part of the tissue chloride is in organic combination.   Peters and Man (9) have found that in lipemic sera a small fraction of the chloride is soluble in petroleum ether, possibly indicating a lipoid-chlorine compound.   However, in the present experi-

tion can only be judged by the reasonableness of interpretations derived therefrom, since tissue cells cannot be directly analyzed. The results must, therefore, be examined to see if they fit in with the distribution of body water and electrolyte as indicated by previous work. Concentrations of cellular electrolyte found may be checked with those expected from osmotic pressure relationships and by comparison with the red blood cell.

The experimental study of the quantitative aspects of the extracellular water volume has received scant attention until recently. Crandall and Anderson (10) estimated the amount of water available for the solution of injected sodium thiocyanate from the concentration of thiocyanate ion in the serum and the quantity of thiocyanate retained. The experiments were carried out on dogs, rabbits, horses, and humans. The results, which varied only slightly in the different animals, suggested that the amount of fluid available for the solution of the thiocyanate ion approximated 30 per cent of the body weight. In man, however, the water available for solution of the thiocyanate ion was somewhat less, ranging from 20 to 25 per cent of the body weight. Lavietes, Bourdillon, and Peters (11) repeated these experiments on humans and also used sucrose in a similar manner. In both types of experiments their values agreed with those of Crandall and Anderson. They suggested that the water volume involved in these experiments represented the extracellular fluid. The volume of extracellular water as obtained in the present study from the concentration and total amount of chloride in the body (Table I, Column 3) agrees closely with the values obtained by Crandall and Anderson and Lavietes, Bourdillon, and Peters. The relatively constant relationship of extracellular fluid to body weight in animals of different sizes and species is an interesting finding. The extracellular fluid of the rat, calculated from the data reported by Light, Smith, Smith, and Anderson (12), has also been found to compose about 29 per cent of the body weight.

The proportion of extracellular water in muscle is of especial

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ments no appreciable amount of tissue chloride was found to be soluble in petroleum ether. In a few analyses of the livers of rats, which we have made, the concentration of chloride was found to be greater than that of sodium. Under some circumstances some of the chloride may not be present as an inorganic salt dissolved in the extracellular fluid.

interest with respect to the problem of the exclusive extracellular distribution of chloride ion.   Muscle was calculated to contain approximately 16 per cent by weight of interstitial fluid.   This was constant for the three animals studied, and similar calculations applied to previous analyses of muscle (13, 14) give identical results.   Eggleton (15) has by another method contributed some data with respect to the extracellular water component of muscle.   When frog skeletal muscle was exposed to solutions of urea of varying concentration, the urea distributed itself throughout all the water of the muscle.   Inorganic phosphate, on the other hand, was found to distribute itself through only 20 per cent of the muscle water.   This was interpreted as the extracellular fraction of the water.   Assuming that the total water content of the muscle was 80 per cent by weight, the extracellular water composed 16 per cent of the muscle weight, which agrees with the values found in the present work.   Hastings and Eichelberger (16) have also concluded from a study of the salt and water exchange between blood and muscle that the muscle cells contain no chloride and that the chloride found in muscle tissue is all present in the interstitial fluid which composes about 20 per cent of the muscle water.

That the distribution of body sodium is closely related to that of chloride is generally accepted.   This association is clearly demonstrated by the results of these studies.   If the sodium of the skeleton is excluded, the tissue sodium in every case except dog muscle can be accounted for by that present in extracellular water.

Although the ash of bone and cartilage has long been known to be rich in sodium (17, 18), the fact that 50 per cent of the body sodium is found in these tissues is surprising.   However, the calculations applied in this study demonstrate that part of the sodium of the skeleton is not analogous to that of other tissues. The compound in which this sodium (the "excess sodium" of the tables) exists is not known at present.   That it functions differently from other tissue sodium seems apparent from the data in Table II.   Since the univalent cations plus the anions with which they are combined are responsible for about ninetenths of the osmotic pressure of the body fluids, it is obvious that for osmotic equilibrium to obtain with the same dissociation

of the univalent cations, the sum of the concentrations of sodium and potassium in the water of the different tissues must be approximately equal to that found in serum water. Examination of the data in Column 12 of Table II shows that for all tissues except the skeleton this is true within the limits of error of the methods employed. In the skeleton, however, the concentration of sodium plus potassium in the total water is greater by about 50 milli-equivalents per liter of water. The concentration of potassium, however, in the intracellular water is approximately equal to that found in the other tissues. The excess sodium must, therefore, contribute little if any to the osmotic pressure of skeletal water and presumably is present as an insoluble or undissociated compound.

Since the analyses did not involve separation of the skeleton into its component parts, it is not possible to state where the sodium is present in greatest concentration. Previous studies by von Bunge (18), Gabriel (17), and, more recently, Logan (19) have demonstrated a high concentration of sodium in the ash of both bone and cartilage. Gabriel was of the opinion that the sodium in bone was associated with the calcium phosphate as an insoluble complex salt. This, however, would not explain the sodium found in cartilage.

The data may be analyzed to see if the amount of excess sodium varies directly with any of the other constituents determined. No constant relationship was found between the sodium and the calcium and phosphorus concentrations. The excess sodium was roughly proportional to the total amount of organic material in the skeleton and to the skeletal nitrogen. Bone and cartilage, particularly the latter, contain relatively large amounts of organic sulfate but this was found not to be sufficient to account for the excess sodium present. In bone the excess sodium is insoluble in water and in strong potassium hydroxide. The compound is soluble in acids, including acetic acid. The sodium in cartilage is water-soluble and is also soluble in acids and alkali.

Whether the small amount of excess sodium found in dog muscle represents intracellular sodium, or whether it resembles that present in skeleton is not known. If present in intracellular water it would amount to 9.5 milli-equivalents per liter. Hastings and Eichelberger (16) have also found more sodium in dog muscle

than can be accounted for by the chloride present. It may be significant that the red blood cell of the dog differs from that of the monkey and rabbit in that it contains sodium as the predominant cation instead of potassium.

In the monkey and rabbit the excess sodium present in the liver may possibly be explained by the inclusion of gallbladder bile in the material analyzed.

From the data presented the concentration of cations in tissue cells can be estimated more closely than has been possible before. The chief intracellular cations are potassium and magnesium. Sodium, as has been pointed out, is almost, if not exclusively, extracellular. The concentration of base in cellular water is approximately 140 milli-equivalents of potassium plus 35 milli-equivalents of magnesium per liter of water, while that in serum water is about 160 milli-equivalents of sodium plus potassium and 6.5 milli-equivalents of calcium plus magnesium. When the relative amounts of divalent cations are taken into consideration, the concentrations agree with those expected at osmotic equilibrium if the base in cells were dissociated to about the same extent as that in serum.

The anions of intracellular fluid consist mainly of protein, organic phosphates, and bicarbonate. Calculations of the concentration of protein and phosphate in cellular fluid are possible from the data given. The most striking finding is the difference between the muscle cell and the red blood cell with respect to concentration of protein. In this respect the muscle cells are considerably more dilute than the red blood cell, containing only about 300 gm. of protein per liter of intracellular water as compared to 450 gm. for the latter. In previous work the assumption has usually been made that the concentration of protein in the muscle cell was equal to that of the red blood cell, but this can no longer be considered accurate. The other tissue cells are not entirely uniform but approach more nearly the red blood cell in their concentration of protein than does the muscle cell. The extremely high concentration of protein found in the skeleton of the dog and monkey can be explained by the fact that part of this protein is not cellular but is present in the extracellular organic matrix of bone and cartilage. The high concentration of protein in skin would be anticipated from the inclusion of epidermis and some hair.

All of the tissue cells are richer in phosphorus than the red blood cells, as is to be expected, since the chief anion of the red blood cell is chloride, which is present in a concentration of about 80 milli-equivalents per liter of cell water. The concentration of phosphorus in the intracellular water of muscle, however, is found to be consistently less than that of the liver and other viscera, that of the muscle being about 100 mM per liter of water, whereas in the other tissues it averages about 180 mM. This greater concentration of phosphorus may be associated with the larger amount of lipoid phosphorus and nucleoprotein in the liver and other viscera than in the muscle.

TABLE III

Distribution of Water, Sodium, and Potassium in Fetus[*]

Fetus No.	Weight	Per cent of body weight as				K in intracellular water	Total Na	Extracellular Na
		Fat	Total water	Extracellular water	Intracellular water			
	kg.					*m.-eq. per l.*	*m.-eq.*	*m.-eq.*
9	0.34	0.7	87	62	25	137	31.3	30.5
8	0.57	1.3	86	60	26	139	53.5	50.2
4	1.55	3.4	82	53	29	140	136.0	122.0
3	1.62	3.9	81	52	29	142	143.3	126.0
7	2.92	6.7	75	43	32	123	240.4	184.0

[*] Recalculated from the data of Iob and Swanson (20).

The application of the calculations described in this paper to tissue and animal analyses reported in the literature provides interesting results. As an example of the added information which may be obtained, Table III represents the results of the chemical analyses of human fetuses of various weights as reported by Iob and Swanson (20). For the concentrations of sodium and chloride in extracellular water average values of 148 and 120 milli-equivalents per liter respectively were assumed. Most of the water of very young fetuses is found to be extracellular. Even in the practically full term fetus weighing 3000 gm., extracellular water comprises 43 per cent of the body weight as compared to about 27 per cent in adult animals and 23 per cent for adult humans. The concentration of intracellular potassium is

found to be of the same magnitude as in adult animals. The presence of excess sodium can be demonstrated in the larger fetuses, in all probability related to the development of the skeletal structures.

SUMMARY

The whole bodies and certain of the separated tissues of dogs, monkeys, and rabbits were analyzed for water, sodium, potassium, chloride, total phosphorus, fat, and nitrogen. Before sacrificing the animals, blood was taken for analysis for the same substances in serum and red cells.

By assuming that all chloride is dissolved in the extracellular water, and that the concentrations of electrolytes in serum water represent the concentrations in all extracellular water, a system of calculations is developed which permits the estimation of the volumes of intracellular and extracellular fluid in the body and in individual tissues, and the concentrations of solutes in these two compartments of the body water.

The extracellular water was found to compose about 27 per cent of the body weight, while intracellular water varied from 29 to 45 per cent, being lower in the fat animals.

In different tissues varying proportions of extracellular and intracellular water are found, but in analogous tissues of different animals the proportions are about the same.

Except for the skeletal sodium and 10 per cent of the sodium of dog muscle, all of the sodium of the body can be accounted for in the same volume of extracellular water which would contain the body chloride.

It is pointed out that about one-fourth of the total sodium in the body must exist in bone and cartilage either in an insoluble compound or in a compound in which the sodium is not dissociated.

The intracellular base of the various tissues is composed of potassium at a concentration of 140 milli-equivalents and magnesium at a concentration of 35 milli-equivalents per liter of cellular water. Base is distributed between the tissue cells and the extracellular fluid in approximately the same relative concentrations in which it is distributed between red blood cells and

Analyses from the literature lead to similar interpretations when subjected to the same methods of calculation.

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THE STIMULATION OF YEAST PROLIFERATION
BY PANTOTHENIC ACID

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Pantothenic acid, discovered by Dr. R. J. Williams, has been shown to stimulate the growth of a number of strains of yeast (1). The amount of the stimulation depended on the strain of yeast used and some of the yeasts grew still more rapidly when inosite or crystalline vitamin B_1 or both were also present in the culture medium (2). Dr. Williams has generously shared with me some of the pantothenic acid in the form of its calcium salt (10 per cent calcium pantothenate), and, while this is not a pure substance, the pronounced effects of it invite experimental analysis.

The yeast used in the following experiments is a pure strain of *Saccharomyces cerevisiæ*, Hansen obtained from a single cell isolation in 1928 and which has given consistent growth since then (3).[1] Williams' culture medium was used and the methods and technique employed have been described in detail elsewhere (4).

Concentrations from 0.05 to 1 microgram of pantothenic acid were added to 10 ml. portions of Williams' original medium, and the yeast growth was measured by counting the number of cells per unit volume of medium or by measuring the density of the population with a photoelectric nephelometer.

The early growth was greatest with a concentration of 0.2 microgram per ml., but a slightly higher concentration of 1 microgram prolonged the stimulation, as shown in Table I. Smaller amounts of the pantothenic acid were inadequate and greater concentrations seemed to be toxic. The addition of 1 microgram of inosite and 0.1 microgram per ml. of pantothenic acid gave an increased growth of 25 per cent at 22 hours after seeding. The

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[1] This yeast isolation is yeast No. 4360 in the American Type Culture Collection.

addition of 1 microgram per ml. of thallium acetate to the above amounts of inosite and the acid decreased the yield of yeast by 5 per cent. While many concentrations were used in the experiments, only those referred to in this paper are included in Table I.

In view of the possible importance of other elements Williams modified his formula by adding small amounts of those elements known to be important to the growth of yeast (2). When the culture fluid is made with Merck's asparagine, as in these experiments, the addition of thallium is less important than when other brands of asparagine are used (5). Therefore, the modified

TABLE I

Yield of Yeast As Percentage of Control Cultures Measured with a Photoelectric Nephelometer

	Original formula				Amplified formula			
Pantothenic acid, *microgram*	0.1	0.2	1	0.1	0	0	0.5	0.5
Inosite, *microgram*	0	0	0	1	0	0	0	0
Thallium, *microgram*	0	0	0	0	0	1	1	0
hrs.								
22	124	174	126	155	118	121	176	109
32	99	128	111	125	102	100	127	94
45	102	124	120	102	104	104	118	109
69	97	116	145	103	106	101	123	112
74	100	103	113	101	104	103	111	101
117	100	101	118	113	107	110	115	107
142	101	103	117	97	100	95	110	105

formula was made both with and without thallium for the further assay of the effect of the pantothenic acid.

A small increase in yield of yeast is obtained with the modified formula but without the thallium (Table I). Adding thallium acetate (1 microgram per ml.) gave an increased yeast growth at first but slightly less growth toward the end of the cycle of population growth. A significant difference should be about 5 per cent or greater, as the experimental error is nearly 4 per cent.

When the thallium is omitted from the modified formula, more pantothenic acid is required for a comparable stimulation of the yeast growth. The addition of 1 microgram per ml. of pantothenic acid to the modified medium without the thallium increased

the yield of yeast by 23 per cent at 22 hours after seeding the population. One-half of the amount of pantothenic acid (0.5 microgram per ml.) gave an increased growth of 176 per cent of that of the control when the complete modified formula was used (including 1 microgram per ml. of thallium acetate). 2.5 times as much pantothenic acid is necessary to obtain the same increase of yeast growth with the modified formula as with the original formula. The growth of yeast is dependent on mineral nutrients as well as on organic materials and the effect of the latter must be separated from the former. This will require a knowledge of the impurities contained in the so called reagent grade of chemicals used in the culture fluids.

The strain used in these experiments is not quite as sensitive to pantothenic acid stimulation as some of the strains used by Williams and Saunders (2), which is not surprising, because it is well known that different yeasts have different nutritional requirements. It is not possible to compare the results directly with those of Williams and his associates, because the criteria used to measure the yeast growth are not identical. A thermocouple was used by Williams for the estimation of the density of his yeast suspensions and his nephelometer was calibrated to measure the yeast growth as mg. of moist weight per unit volume of medium. This is a valid criterion for the measurement of yeast growth, but it cannot be duplicated in other laboratories unless the optical condition of the yeast and the amount of moisture are known (6). To show this difference I calibrated my nephelometer in terms of moist yeast, using a bit of yeast from the center of a Fleischmann's cake. With this moist yeast criterion the maximum percentages of increased yeast growth (Table I) become 206 and 208 instead of 174 and 176.

When the growth of yeast is measured in terms of the number of cells present in a unit volume of the culture medium, the stimulation of the growth by pantothenic acid is shown to be slightly greater than when measured nephelometrically. As the effects with the different media were relatively the same, the number data are not here given. The use of two criteria for growth shows that the distribution of cells of different size and the optical density of the cells are different when the growth is stimulated by pantothenic acid from those of the control cultures without the

added acid. The evaluation of the effects of substances on the growth of yeast necessitates the use of more than one criterion for the measurement of the growth and the interrelations between the different criteria used. A critical study of this problem is under way and will be published in the near future.

The stimulating effect of the pantothenic acid on the growth of yeast decreases as the populations grow and Williams and his associates have emphasized the importance of measuring the effect during the early growth. The smaller the amount of the acid added the sooner will its effect disappear. The maximum amounts of stimulation occurred in my experiments from 18 to 24 hours, which is in good agreement with the observations of Williams.

Some of my experiments failed to show stimulation by the addition of pantothenic acid. When the seed yeast of the strain used is from a rapidly growing population that has not been restricted by the environment, there is very little stimulation of the growth by the pantothenic acid. The greatest stimulation occurs when old cells are used for the seed yeast. Cells from a population 7 days old gave pronounced effects. Such cells were beginning to change into the resistant cells, and when they are planted into the fresh medium there is a period of time, lag period, before the cell proliferation becomes logarithmic (7).[2] The length of the lag period depends on the age of the seed yeast and when the seed yeast is growing logarithmically there is, of course, no lag period.

When pantothenic acid is present in the medium, the lag period is shortened, demonstrating that the recovery of the yeast is facilitated. Whether this is a stimulation of budding or the furnishing of an essential material cannot be determined until pure preparations of the acid become available.

The relative rate of yeast population growth (increase in yeast per unit of time per unit of yeast) is greater when the optimum amount of pantothenic acid is present in the medium. The difference between the relative growth rates with the acid and of the control culture may be as great as 81 per cent during the first 11 hours of population growth. Later the rate approaches

[2] The seed yeast used by Williams and Saunders (2) may have been aged somewhat, because it was subjected to cold to aid in obtaining a uniform suspension of seed yeast. (Personal communication from Mr. Saunders.)

and becomes, within the experimental error, the same as that of the control culture. During the period of increased growth the generation time is less, so that the increased growth is due to more rapid cell division.

The ultimate yield of yeast is the same as that of the control populations. The pantothenic acid does not lessen the progressive limiting of the growth by the increasingly unfavorable medium. When old cells are used as the seed yeast, the final yield is slightly greater than that of the control when the pantothenic acid is added. Thallium alone has little effect on the relative rate of growth but the period of logarithmic growth is prolonged. The pantothenic acid effect is more pronounced during early growth and the combination of both in the modified formula leads to a slight, but not marked, increase of yeast. The addition of thallium to the pantothenic acid and inosite in the original formula decreased the growth throughout the growing period.

SUMMARY

Small amounts of pantothenic acid concentrate (0.2 microgram per ml. with Williams' original formula for the medium or 0.5 microgram per ml. with his modified formula) produce a marked increase in the growth of a pure strain of *Saccharomyces cerevisiæ*. The presence of the pantothenic acid increases the yeast growth by lessening the generation time and increasing the relative rate of growth. The increase is more pronounced when the seed yeast comes from old cultures and then the lag period preceding the phase of logarithmic growth is shortened. Little effect occurs with young seed yeast because there is no lag period. The acid has little effect on the crop, or final yield of the yeast, and the maximum stimulation occurs at about 18 to 24 hours after seeding the population. The observations suggest that the pantothenic acid stimulates the recovery of the budding rate of older, differentiated yeast cells, but whether this is a direct stimulation or due to the presence of an essential substance cannot be determined until the acid is available in the form of a pure substance.

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METAL CATALYSTS IN BIOLOGIC OXIDATIONS*

I. THE SIMPLE SYSTEM: THIOGLYCOLIC ACID, BUFFER, METAL, DITHIOL†

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It is well recognized that heavy metal ions play a major catalytic rôle in controlling the course and speed of metabolic reactions, especially oxidations, of various substrates and intermediates, in cells or *in vitro*. The metal ions in the cell, however, are rarely active as such, but form a wide array of complexes, of which some are highly catalytic, others quite inert. For reasons developed more completely in Paper II, we considered it not improbable that the normal balance of cell oxidation might be achieved by the presence in cells of two sorts of substances competing with each other for the metals and forming, respectively, active and inactive complexes. Such augmentor and especially inhibitor substances could easily be demonstrated in tissue extracts if a quantitative method of measuring the catalytic activity of metal ions were available, since the extracts could be added to a standardized system and the change in activity noted.

The pioneer contribution of Warburg (1927), who estimated metal ions in tissue by the effectiveness of its ash in catalyzing cysteine oxidation, has been further exploited by many competent workers, and we had hoped to find such a method applicable to our problem. When, however, the more complex situation met in tissue extracts, as compared to ash, was encountered, it was

soon apparent that a more exhaustive study of the method itself was a necessary preliminary undertaking. When this analysis was largely completed, several careful studies appeared, which our own findings in part confirm; in part, extend.

The present paper reports an exploration of the catalytic action of iron, copper, and manganese ions on the oxidation of thioglycolic
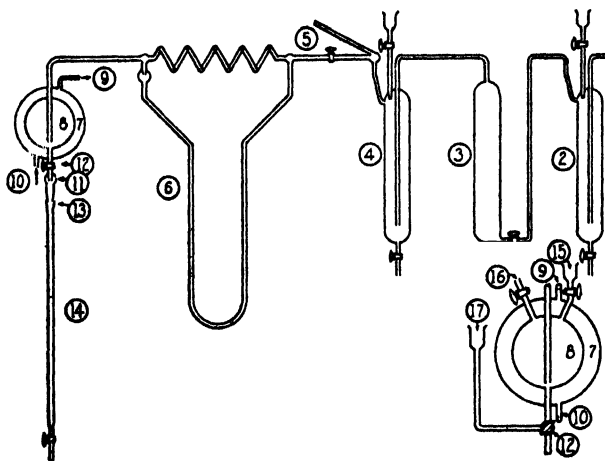
Fig. 1. Apparatus for the titrimetric method of measuring rates of oxidations. *1*, oxygen intake; *2*, alkaline permanganate wash vessel; *3*, finely divided silica gel, held in place by cotton plugs to remove suspended particles; *4*, water wash bottle to resaturate gas; *5*, fine stop-cock to adjust gas flow; *6*, flowmeter containing a good grade of mineral oil and a dye; *7*, outer water jacket to maintain temperature constant (*10* and *9*, intake and return to thermostat); *8*, reaction vessel; *11*, aperture to allow escape of air while burette is filled; *12*, 3-way stop-cock; *13*, ground joint to burette; *14*, 25 cc. burette; *15*, funnel for introduction of materials into reaction vessel; *16*, oxygen exit during reaction; *17*, arm that connects through 3-way stop-cock (*12*) with burette (*14*) and permits washing of latter between titrations.

acid as the following factors are varied: temperature, oxygen tension, initial thiol concentration, initial dithiol concentration, hydrogen ion concentration, character of buffer, especially phosphate and carbonate, combinations of ions, specific ion inhibitors—cyanide, pyrophosphate, and the like. Thioglycolic acid was employed in our studies because it is easily prepared, gives repro-

ducible results, and may be considered as fairly analogous to substances actually participating in important cell oxidation reactions.

Methods

It is apparent that the progress of the oxidation of thioglycolic acid may be followed by measuring the volume of oxygen consumed or the amount of unoxidized thiol remaining. For the former determination, the manometric method is quite satisfactory; for the latter, periodic titrations with a standard iodine solution are required. The reaction involved is

$$2R—SH + I_2 = R—S—S—R + 2HI$$
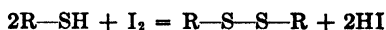
The titrimetric method is more time-consuming than the manometric, since fewer experiments may be performed simultaneously, but it might have proved more specific in the presence of complex substances, as proteins, derived from tissue. We have, therefore, used both methods, though, as it developed, both gave closely concordant results for the systems studied.

Apparatus—For the titrimetric measurements, the oxidation was effected in the apparatus shown in Fig. 1. Manometric measurements were carried out by the method of Warburg (1927), as modified by Gerard (1931).

Reagents—Doubly distilled water was used in the preparation of all solutions, as well as in the crystallization of solid reagents. Liquids were also distilled twice before use. All reagents were stored in Pyrex bottles provided with ground glass stoppers. The reagents were tested chemically for metal traces, with negative results. (Reagents stored in soft glass bottles for relatively short times give abnormally high rates of oxidation of thioglycolic acid.) The thiol itself was kept in sealed Pyrex bulbs, a diluted stock solution being kept no longer than 1 month. The great sensitivity of the thiol to oxidation in the presence of minute amounts of heavy metal ions (about 1 gm. atom of copper or manganese in 2 million cc. of solution) makes it imperative to take all possible precautions against contamination.

The metal catalysts were introduced in the form of dilute solutions of the sulfates (ferrous ammonium sulfate in the case of iron). The carbonate buffers were dilute solutions of sodium carbonate. Other buffers were prepared according to the directions of Clark (1928).

Treatment of Experimental Data—The data obtained for the metal-catalyzed oxidation of thioglycolic acid do not fit the usual reaction equations. The rate at which the thiol is oxidized, however, is linear for most of the reaction (from 5 to 50 per cent oxidation at least), so that (d moles R—SH)/dt is constant during this time.[1] The oxidation rate is, therefore, concisely expressed by a single number, the slope of this line. To avoid fractions, moles of thiol are multiplied by 10^6; time is in minutes.

The manometric data are expressed as usual in c.mm. per unit of time, though the unit chosen here is 100 minutes rather than 1 hour. As above, only the linear portion of the oxidation is used to obtain a value. Results are reproducible within ± 0.02 c.mm.; and we have, in fact, found it satisfactory to calibrate new manometer vessels by charging them with a known amount of thiol and tipping in copper after equilibrium is reached in the thermostat. Although the two notations do not give directly comparable numbers, this is irrelevant for most purposes, as complete experiments were performed in both cases.

Results

Temperature and Oxygen Concentration—Fairly complete data on the influence of temperature and oxygen concentration on this oxidation are available (Elliott (1930); see also Elvehjem (1930) on cysteine oxidation); therefore our own similar findings are briefly summarized.

Under similar conditions, the rates of oxidation of 0.014 M thioglycolic acid at 20° and 1.6° are 116 and 52, respectively (rate in moles $\times 10^6$ R—SH oxidized per minute, carbonate buffer at pH 8.7; 10^{-5} M Cu^{++}). Since a decrease in temperature of 18° is accompanied by a decrease of 55 per cent in rate, the change is roughly 3 per cent per degree. Obviously a temperature fluctuation of $\pm 0.1°$ does not affect results.

Fig. 2 illustrates the influence of oxygen concentration and rate of flow upon thiol oxidation (at 20°, other conditions as above). It is interesting that, below optimal concentration and attendant

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[1] This constancy, also noted by Elliott (1930), suggests that metal and thiol react at once to give a fixed amount of active catalyst which does not decompose as thiol is lost; or that some accelerating factor accumulates as thiol decreases.

maximal constant rate of oxidation, changes in the concentration of dissolved oxygen markedly affect the oxidation rate, even though diffusion or mixing with the reactants is not a factor.   With pure oxygen, 4 liters per hour permits maximal rates.   Extrapolations of the three curves, for different oxygen supply, cross the time axis at the same point, indicating a lag of 2 minutes.   This can hardly be time required for solution of oxygen to the equilibrium value, for the solution is in equilibrium with the gas before the catalyst is added.   More probably during the induction period an active catalytic substance is being formed.

Initial Thiol Concentration—To test for metal catalysis, low
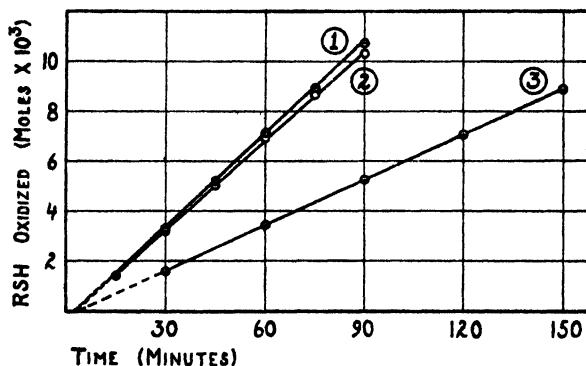
Fig. 2. Influence of oxygen supply upon the rate of oxidation of 0.014 M thioglycolic acid at 20° in carbonate buffer in the presence of cupric ions (9.5×10^{-6} M).   Curve 1, oxygen supplied at the rate of 8 liters per hour; Curve 2, oxygen at 4 liters per hour; Curve 3, air at 4 liters per hour.

thiol concentrations are preferable to high because of the greater sensitivity of a dilute system to small absolute amounts of metal ions, and because in more concentrated mixtures metal salts are likely to precipitate with buffer or other radicals.   A test of high absolute sensitivity is desirable for the small quantities of the extracts available from tissues.   Oxidation rate increases with thiol concentration, but very slightly when the concentration is high enough, presumably, to keep the catalytic ions almost continuously bound.

Our results (Table I) confirm the observation of Elliott (1930) that, in the presence of copper ions, the rate of oxidation increases with the initial thiol concentration, more rapidly at low concentra-

tions than at high. The most dilute solution is completely oxidized in 100 minutes, the most concentrated only 27 per cent, so that the former affords a more sensitive test for metals; and in anticipation of later work we have chosen a fairly dilute thiol for our regular procedure.

From the difference in rates for the two lowest initial thiol concentrations recorded in Table I, it may be calculated that a change of 1 per cent in initial concentration leads to a change of only 0.3 per cent in rate. Accordingly, even a 10 per cent error in concentration would affect the rate by only 3 per cent, which is within the limit of accuracy set by other factors.

Effect of Hydrogen Ion Concentration. Influence of pH on the Stability of Dithioglycolic Acid—In solutions more alkaline than

TABLE I

Effect of Initial Concentration of Thioglycolic Acid on Rate of Oxidation

Temperature, 20°; 10^{-5} M cupric ions; carbonate buffer, pH 8.7; oxygen supplied at 4 liters per hour.

Initial thiol concentration	Rate $\dfrac{\text{Moles R–SH oxidized} \times 10^6}{\text{Time in min.}}$
M	
0.200	54
0.050	22
0.025	16
0.012	12

pH 9, the dithiol formed becomes unstable and is further oxidized, so that oxidation rates are not fully comparable with those in the more acid range and the total oxygen used exceeds the theoretical requirement for the simple equation

$$2R–SH + \tfrac{1}{2}O_2 \rightarrow R–S–S–R + H_2O$$

To study the stability of dithioglycolic acid in alkaline solutions, a known amount of the thiol was oxidized without decomposition at pH 6.0, the oxygen was removed by prolonged passage of nitrogen (rendered oxygen-free by forcing it through a porcelain candle in alkaline sodium hydrosulfite), and, without interrupting the nitrogen stream, the solution was brought to pH 8, 9, or 12, in separate tests, by adding oxygen-free alkali solution. Samples were then drawn from the reaction bulb at intervals and titrated

in the usual manner.   At pH 12, the solution reduced a steadily increasing amount of iodine; at pH 8 or 9, a small constant blank titration was obtained; so that only at high pH does the dithioglycolic acid progressively decompose to form reducing substances.

In another experiment, thioglycolic acid, in the presence of cupric ions, was oxidized as usual, but at pH 12, and the iodine titrations were continued for 3 hours.   The minimum titer was
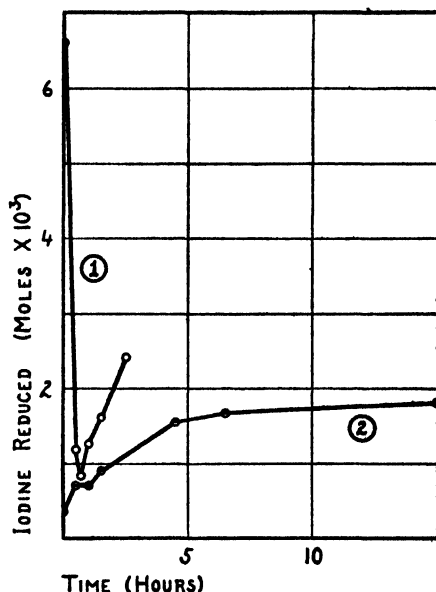
FIG. 3. The instability of dithioglycolic acid at pH 12.   Curve 1, 20°, 4 liters of oxygen per hour; initial concentration of thioglycolic acid, 0.014 M; 10⁻⁵ M cupric ions; Curve 2, the same, except that oxygen was replaced with nitrogen.

reached in less than 1 hour, after which it rose (Fig. 3), again showing the instability of the dithiol at this pH (Wieland and Franke, 1928; Dixon and Tunnicliffe, 1923).

Influence of pH on Metal Catalysis of Thiol Oxidation—Since the addition of buffers is essential in any study of the influence of pH, it is sometimes difficult to discriminate between the effect of hydrions *per se* and of the buffer ions.   This is particularly true in the present study since, as will be shown, the usual buffers pro-

foundly influence the catalytic activity of all the metal ions, and no one buffer covers a sufficiently extensive pH range. The catalytic rates with copper, however, were identical in such differ-

TABLE II

Influence of pH on Copper Catalysis of Thiol Oxidation (Titrimetric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; 10^{-5} M cupric ions; oxygen supplied at 4 liters per hour.

Buffer*	pH	Rate Moles R—SH oxidized × 10^6 / Time in min.
Carbonate†	3.0	0
Phthalate	5.0	4
"	6.0	16
Orthophosphate	6.0	16
"	7.0	11
"	8.0	11
Borate	8.0	10
"	9.0	13

Buffer	Catalyst, M × 10^6	pH	Rate
Orthophosphate	7 Fe++	6.0	84
Borate	7 "	9.0	21
Carbonate	7 "	8.2‡	43
"	7 "	8.7	60
"	5 Mn++	8.2	74
"	5 "	8.7	92

* With the exception of the carbonate, all buffers were prepared according to the directions of Clark (1928). The final crystallizations of the salts were made with the use of doubly distilled water.

† In this experiment the system can scarcely be properly said to have been buffered. Enough Na_2CO_3 was added to bring the system to pH 3.15. After 90 minutes no oxidation had occurred, and the final pH was 3.20.

‡ To maintain the pH at 8.2 it was found convenient to pass from 7 to 10 per cent of the oxygen through a saturated bicarbonate solution at 20°. Otherwise, the pH of a carbonate-buffered system rapidly rises under these conditions from an initial value of 8.2 to 8.7, where it remains constant.

ent buffers as orthophosphate and phthalate at pH 6, and agreed within 10 per cent at pH 8 in orthophosphate and borate, so that it may reasonably be assumed that differences evident through the pH range are largely dependent upon the changing hydrion

concentration. The rate of oxidation of thioglycolic acid in the presence of copper was determined at pH values of 3, 5, 6, 7, 8, and 9. The rate increases from negligible values at pH 3 to a maximum at 6, is diminished at pH 7 and 8, and at 9 rises toward the maximum at 6 (Table II). The complication introduced by specific buffer effects in manganous and ferrous ion catalysis is indicated in the lower section of Table II. In carbonate, rates for each ion increase with alkalinity between pH 8 and 9.

Buffers—The influence of some common buffers (especially those containing ions encountered in tissue preparations) on the various metal catalyses was rather fully examined. Comparisons of two were, of course, performed at the same pH, and most experiments were carried out at pH 8 to 8.6. In certain cases the pH tended to drift toward the alkaline side during a run, giving a positive error in the individual sample but not invalidating the general relations.

Carbonate—The changes produced by various phosphates on metal catalyses are referred in the following discussion to the metal activities in carbonate as a standard. It is difficult to justify fully the choice of carbonate buffer as a reference point, since the carbonate ion may exert a specific effect, just as do the phosphates. However, from data on hand (Tables IV, V, *a*, VI), we believe that catalytically the carbonate ions are relatively inert. True, the lower activity of copper in carbonate than in pyrophosphate (Table III) may represent depression by the former rather than acceleration by the latter; but, since the effect of pyrophosphate varies with its concentration and that of carbonate does not, it seems reasonable to attribute the specific action to the pyrophosphate. Carbonate is in general preferable for the study of tissue catalysts; the disadvantages of phosphate are indicated in more detail in the next section, and borate, while permitting the catalytic action of all these metals (Warburg, 1927), is difficult ·to free of its metal impurities, and also forms a variety of organic complexes which might disturb results with tissue extracts. The buffer action of carbonate is not very strong at pH 8, but in experiments in which it is used the pH rarely altered over 0.1 unit during the run, an inconsiderable change.

The interactions of metal ions in carbonate buffer are strikingly different from those in phosphate. Copper and iron, and man-

ganese and iron give simple additive effects when present together; while copper and manganese together exhibit a tremendously increased action.[2]   Thus, a rate of thiol oxidation of unity in the presence of 1 unit of copper is more than doubled by the addition of 0.1 unit of manganese, itself only one-third as active as copper (see Table VII, *a*).   Since small amounts of manganese added to larger quantities of copper produce a greater acceleration than the converse system, it seems probable that the manganese acts as accelerator to the copper.

Phosphates—Since Warburg (1927) early found that pyrophosphate markedly affected the oxidation of cysteine under metal catalysis and much other work indicates an important rôle of phosphates in biologic oxidations, the sodium salts of ortho-, meta-, pyro-, and certain organic phosphates were studied in relation to the catalytic influence of copper, iron, and manganese on the thiol oxidation.   The catalytic action of iron (assuming activity in carbonate buffer as standard) is decreased about 50 per cent by orthophosphate.[3]   This effect is but little greater when the molar ratio of phosphate to ferrous ion is 10,000 than when it is 100 (see Table III, second column).   Results with metaphosphate were less regular but also indicate a depression of about 40 per cent in the catalytic activity of iron when the salt is present in molar ratios of from 10 to 10,000.   Most inactivating is pyrophosphate, which, even when present in the low ratio of 4 moles to 1 of iron, gives a 60 per cent lowering, and in a ratio of 5000:1 suppresses 90 per cent of the iron catalysis.

Manganese is still more sensitive to the various phosphate ions, all of which depress its action progressively with increasing concentration.   Thus for orthophosphate the activity loss is 25 per cent at a molar ratio of 100 and 91 per cent at 10,000; for metaphosphate, 30 per cent at 4, 92 per cent at 160, 96 to 99 per cent at 3000.

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[2] Warburg (1931) reports a somewhat similar interaction between iron and manganese for the oxidation of cysteine in alkaline borate solution with cyanide present.

[3] Orthophosphate contains metal impurities that cannot be removed by repeated crystallization but which do settle from a stock solution after a week or more as insoluble complexes.   Lower rates are therefore obtained in "aged" solutions.   We have used such solutions since the situation became clear.

<div align="center">

TABLE III

Influence of Phosphates on Metal Catalysis of Thiol Oxidation (Titrimetric Measurements)

</div>

Temperature, 20°; 0.014 M thioglycolic acid; sodium carbonate stock solution used to adjust pH; initial pH 8.2 in all cases; oxygen supplied at 4 liters per hour. Rate calculated in terms of (moles of R—SH oxidized $\times$ 10⁶)/(time in minutes).

	Molar ratio, phosphate to metal ion	Rate			Final pH
		Fe^{++} $(4 \times 10^{-5} M)$	Mn^{++} $(5 \times 10^{-4} M)$	Cu^{++} $(5 \times 10^{-6} M)$	
Orthophosphate	0:1*	(43)†	74	38	8.2
	100:1	21	55		8.3
	1,000:1	20	34		8.3
	2,500:1	20	21		8.2
	5,000:1	15	13		8.2
	10,000:1	17	7	48	8.2
	20,000:1		7		8.2
Metaphosphate	0:1*	(43)†	74	38	8.2
	100:1	22	67	112	8.6
	500:1		27		8.7
	1,000:1	14	10	126	8.7
	10,000:1		4	182	8.6
Pyrophosphate	0:1*	(43)†	74	38	8.2
	4:1	15	32		8.8
	8:1		20		8.8
	16:1	11	10	66	8.8
	160:1	13	6	60	8.6
	1,330:1			69	8.5
	2,660:1		1	70	8.4
	3,530:1		3		8.4
	5,320:1	3		78	8.4
	21,250:1			112	8.3
	42,500:1	3	3	113	8.2

* Refers to the rates obtained in a carbonate buffer.

† The rate characteristic of this concentration of iron ions was not determined at pH 8.2. However, at double this concentration ferrous ion is inhibited to the extent of 42 per cent in a phosphate buffer as compared to carbonate.

The activity of copper, on the contrary, is increased by these buffers. Pyrophosphate in a molar ratio of 16 gives a 65 per cent increase in the catalytic effect; of 2500, 80 per cent; and of 40,000,

190 per cent.[4] Orthophosphate acts in a similar, though less powerful, manner; and metaphosphate may be even more effective.[5] Experimental values are summarized in Table III.

It may well be emphasized that these results cannot be transferred freely from one substrate to another. For example, pyrophosphate does not augment but depresses the action of copper in catalyzing the oxidation of carbohydrates (Krebs, 1927).

Sodium Glycerophosphate—Sodium glycerophosphate (also, in a few experiments, hexosemonophosphate) is inactive or slightly accelerating as regards iron and manganese catalyses, although

TABLE IV

Influence of Sodium Glycerophosphate on Thiol Oxidation by Metals

Temperature, 20°; 0.014 M thioglycolic acid; oxygen supplied at 4 liters per hour. Rate calculated in terms of (moles of R—SH oxidized $\times 10^6$)/(time in minutes). Catalysts, 5×10^{-6} M Cu^{++}, 8×10^{-5} M Fe^{++}, 5×10^{-6} M Mn^{++} ions.

Buffer	pH	Addition	Catalyst	Rate
Carbonate	8.2		Cu^{++}	38
"	8.2		Fe^{++}	43
"	8.2		Mn^{++}	74
"	8.2	0.02 M glycerophosphate		3
"	8.2	0.02 " "	Cu^{++}	46
"	8.2	0.02 " "	Fe^{++}	47
"	8.2	0.02 " "	Mn^{++}	84
"	8.2	0.02 " orthophosphate	Fe^{++}	25
"	8.2	0.02 " "	Mn^{++}	14
Orthophosphate (0.02 M)	8.1	0.02 " glycerol	Fe^{++}	20

the glycerol alone, as well as the orthophosphate, is inhibitory. Aside from the chemical significance of such findings, they can hardly fail of considerable biologic significance, since the normal metabolic traffic in tissues involves continuous formation and loss of ortho-, pyro-, and several organic phosphates, with a probable consequent shift in catalytic activity of metal ions.

Table IV illustrates the difference in action of sodium glycero-

[4] Compare with similar findings on cysteine oxidation (Elvehjem, 1930).

[5] Elliott (1930) reports an inhibitory effect of orthophosphate on copper catalysis.

phosphate and sodium orthophosphate on iron and manganese catalyses.

Effect of Metal Ion Concentration—The marked increase of catalytic effect with increasing copper concentration, in pyrophosphate buffer is shown in Fig. 4.   Data for other ions and buffers as well are given in Tables V, V, *a*, and VI.   The catalytic activity of copper in pyrophosphate buffer, as measured by the rate of thiol oxidation, is not a linear function of the metal concentration, but is roughly proportional to its square.
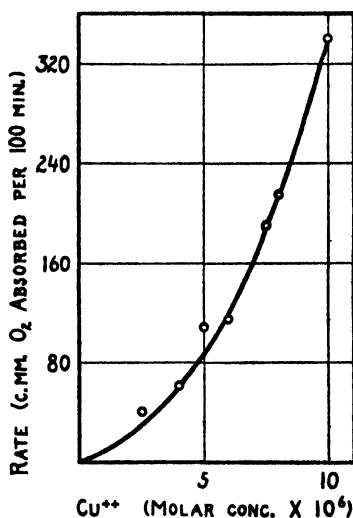
Fig. 4. Effect of cupric ion concentration on the rate of oxidation of thioglycolic acid in pyrophosphate buffer at 20°.

Mutual Interaction of Metal Ions—It is significant that iron and manganese, although almost inactive individually in a pyrophosphate buffer, are still able to influence the action of copper.   Manganese depresses the catalytic activity of copper by 20 to 30 per cent, while iron increases it by 10 to 20 per cent.   When both ions are added to copper, the inhibitory effect prevails.   Again the nature of the substrate appears important, since, for cysteine oxidation, Warburg (1927) found no interaction between copper and manganese.   In carbonate, manganese and copper together have an increased catalytic activity.

TABLE V

Effect of Metal Ion Concentration on Rate of Oxygen Absorption by Thiol in Pyrophosphate (Manometric Measurements)

Temperature, 20°; 0.05 M sodium pyrophosphate buffer; pure oxygen atmosphere; original thiol concentration, 0.014 M.

Cu++		Mn++		Fe++	
M × 10⁶	O₂ absorbed per 100 min.	M × 10⁶	O₂ absorbed per 100 min.	M × 10⁶	O₂ absorbed per 100 min.
	c.mm.		*c.mm.*		*c.mm.*
2.5	40	2	3	10	2
4.0	61				
5.0	108	10	3		
6.0	115			20	3
7.5	190	20	3.5		
8.0	215			50	4
10.0	340	100	1		

TABLE V, *a*

Effect of Buffers and Metal Concentration on Rate of Thiol Oxidation (Titrimetric Measurements)

Temperature, 20°; initial thiol concentration, 0.014 M; oxygen supplied at 4 liters per hour. Rate calculated in terms of (moles of R—SH oxidized × 10⁶)/(time in minutes).

Buffer, 0.002 M	Catalyst, M × 10⁶	pH	Rate
Phthalate	5 Cu++	5.0	11
"	10 "	5.0	28
"	5 "	6.0	80
"	10 "	6.0	160
Orthophosphate	5 "	6.0	77
"	10 "	6.0	174
"	5 "	8.0	48
"	10 " .	8.0	104
Borate	5 "	8.0	45
"	10 "	8.0	99
Orthophosphate	7 Fe++	6.0	84
"	14 "	6.0	190
Borate	7 "	9.0	21
"	14 "	9.0	42

Tables VII and VII,*a* record the experimental results obtained by the manometric and titrimetric methods respectively. For convenient comparison, calculated additive rates from Tables V and VI are included.

Effect of Dithiol—Harrison (1927), following the lead of Dixon and Tunnicliffe (1923), has demonstrated an autocatalytic oxidation of thiols in the absence of heavy metals. The oxidation follows a sigmoid curve and is definitely accelerated, in the early stages, by addition of dithiol. Gerwe (1931) likewise has obtained autoxidation of cysteine under presumably metal-free conditions (but see Elvehjem, 1930). The catalytic action of the dithiol is, however, greatly enhanced in the presence of metals, so that we

TABLE VI

Effect of Metal Concentration on Rate of Thiol Oxidation in Carbonate Buffer
(Titrimetric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer; oxygen supplied at 4 liters per hour. Rate calculated in terms of (moles of R—SH oxidized $\times$ 10^6)/(time in minutes).

Catalyst, M $\times$ 10^6	pH	Mean rate	No. of experiments in mean	Maximum variation from mean
				per cent
5 Cu^{++}	8.2	38	3	8
80 Fe^{++}	8.2	43	3	7
5 Mn^{++}	8.2	74	2	2
5 Cu^{++}	8.7	49	10+	9
10 "	8.7	122	8	5+
20 "	8.7	266	1	
40 Fe^{++}	8.7	22	7	8+*
80 "	8.7	60	3	10 -
0.3 Mn^{++}	8.7	11	10+	8
0.6 "	8.7	21		
1.2 "	8.7	28		
5.0 "	8.7	92		

* Does not include three results that deviate greatly from the mean.

have been able to note distinct effects with relatively small (compared to Harrison's experiments) proportions of dithiol added to the thioglycolic acid.

As could now be anticipated, the dithiol influence varies according to the metal added, the buffer, etc., but under all conditions tried it is in the positive direction. 1 part of dithiol added to 10 of thiol, for example, leads to the following increases in rate of oxidation; for copper in orthophosphate 30 per cent, copper in carbonate 56 per cent, iron in orthophosphate 28 per cent, iron in

carbonate 19 per cent. It will be noted that the increased activity induced by the dithiol for each metal ion is greatest in the buffer in which the initial activity is least. Along the same line, the

<div align="center">TABLE VII</div>

<div align="center">Effect of Iron and Manganese Ions on Copper Catalysis in Pyrophosphate
(Manometric Measurements)</div>

The experimental constants were the same as for Table V.

Catalysts (M × 10⁶)			O₂ absorbed per 100 min.	
Cu⁺⁺	Mn⁺⁺	Fe⁺⁺	Calculated, additive	Observed, combined
			c. mm.	*c. mm.*
7.5	10.0		193	140
10.0	10.0		343	280
7.5	20.0		194	140
8.0	100.0		216	142
10.0	20.0		343	260
10.0		10.0	342	410
10.0		20.0	344	400
8.0		50.0	219	252
7.5		10.0	192	210
7.5		20.0	194	220
10.0	2.0	20.0	347	310
8.0	2.0	50.0	223	174

<div align="center">TABLE VII, a</div>

<div align="center">Mutual Augmentation of Catalytic Activities of Copper and Manganese in
Carbonate Buffer (Titrimetric Measurements)</div>

The experimental constants were the same as for Table VI.

Catalysts (M × 10⁶)		Rate	
Cu⁺⁺	Mn⁺⁺	Calculated, additive	Observed, combined
5.0	5.0	141	202
5.0	1.2	77	155
5.0	0.6	70	109
5.0	0.3	60	76

activity of manganese, largely lost in orthophosphate, is mostly restored by the addition of dithiol. In carbonate, there is a much smaller percentage increase, though the absolute effect is about the same as in phosphate.

TABLE VIII

Effect of Dithiol on Rate of Oxidation of Thiol by Metals in Orthophosphate and Carbonate Buffers (Titrimetric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; oxygen flow, 4 liters per hour.   Rate = (moles of R—SH oxidized × 10^6)/(time in minutes).

Buffer	Dithiol concentration M × 10^4	Rate			
		No metal added	Cu^{++} (5 × 10^{-4} M)	Fe^{++} (8 × 10^{-5} M)	Mn^{++} (5 × 10^{-4} M)
Orthophosphate, pH 8.1	0	1	56	25	8
	14		63	32	
	35		71	41	17
	70		90	60	32
	140	9			
Carbonate, pH 8.2	0	1	38	43	74
	14		64	55	
	28			64	
	42			71	
	70		73	99	95
	140	9			

FIG. 5. Influence of addition of dithioglycolic acid to a thioglycolic oxidation system in orthophosphate buffer.   Temperature, 20°; iron catalyst (8 × 10^{-5} M); initial concentration of thioglycolic acid, 0.014 M.   Ratio of dithiol to thiol, Curve 1, 1:2; Curve 2, 1:4; Curve 3, 1:10; Curve 4 no dithiol.

When the amount of dithiol added is varied, further differences emerge. Thus, for copper, the accelerating effect of dithiol is about proportional to its concentration in orthophosphate buffer, but in carbonate small amounts are relatively more effective. In the case of iron, the dithiol effect varies with its concentration in both buffers. Table VIII illustrates the marked acceleratory influence of dithioglycolic acid on the metal catalysis of thioglycolic acid oxidation.

In Fig. 5 the influence of the addition of various ratios of dithiol to an iron-catalyzed orthophosphate thiol oxidation system is graphically illustrated. The slopes of the curves are, of course, proportional to the respective rates of oxidation.

The degree of change with inhibitor and accelerator substances will depend on the relative concentrations of thiol and active substance, the relative stabilities of the metal complexes formed with each, and the relative catalytic effectiveness of the complexes formed. When the first two relations are favorable and the third positive, that is, when the new complex is more effective as a catalyst than that with the thiol, then acceleration results, as with dithiol. When the new complex is less effective or inactive, inhibition occurs as in the classical case with cyanide.

SUMMARY

1. The catalyzed oxidation of thioglycolic acid by oxygen in the presence of metal ions has been studied by the manometric measurement of oxygen used and by iodine titration of the thiol remaining.

2. Results obtained as temperature and oxygen content are varied are summarized.

3. The influences of hydrion concentration and specific buffer effects were examined, especially on copper catalysis. With this ion, the rate increases from 0 at pH 3 to a maximum at 6, falls again to 8, and rises sharply at 9. At greater alkalinity the disulfide undergoes oxidative decomposition.

4. Carbonate buffer has little specific influence on the catalysts studied. In this, combinations of iron with copper or manganese show an activity equal to their summed separate actions. Copper and manganese, however, are tremendously more active together than apart.

5. Ortho-, meta-, and pyrophosphates exert marked influences on iron, manganese, and copper catalyses. The three inhibit iron with increasing power in the order given, small amounts of buffer being almost as effective as much larger quantities. Manganese is even more sensitive, the various phosphates inhibiting progressively more with increasing concentration. Copper, on the other hand, is accelerated by the phosphates, activity increasing roughly as the square of the metal ion concentration.

6. Buffers made from glycerophosphate and hexosephosphate do not affect catalyses by iron and manganese.

7. Copper catalysis in phosphate buffers is decreased by the simultaneous presence of manganese, increased by iron.

8. Dithiol, present at the start, hastens thiol oxidation by all metals and in all buffers used. Special quantitative relations are described in the text.

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METAL CATALYSTS IN BIOLOGIC OXIDATIONS*

II. TISSUE INHIBITORS†

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The rate at which a tissue burns substrate and frees energy is,
for it, a basic datum and may be said to measure its "speed of
life" (Gerard, 1932). This rate, though subject to great increase
during activity, is remarkably constant under ordinary condi-
tions of rest. Yet little attention has been paid to the quantita-
tive control of oxidations at rest, and less to the problem of an
increase during function. If the skeleton reaction

$$\text{Substrate} + \text{oxygen} + \text{catalyst} \rightarrow \text{oxidized products}$$

be considered, it is easily seen that the reaction velocity, according
to the mass action law, might vary with the concentration of
reactants and products. In extreme cases it is true that these
may play a critical rôle, but ordinarily they do not. Thus, when
the oxygen concentration is greatly diminished, cell respiration
begins to decrease; when substrate is withheld, especially for
simple bacterial cells, the same is true; and when CO_2 or other acid
is allowed to accumulate in excess, respiration is again slowed.
Yet under conditions approaching normal, any or all of these
factors may be widely varied without altering the respiratory rate
of the resting cell. The critical factors must be, then, the amount

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of Chemistry of the University of Chicago.

of active catalytic system and the conditions determining its accessibility to substrate, degree of activity, etc.

As oxidation catalysts, metal complexes, especially the iron porphyrins, are recognized as playing an outstanding part. Though attempts to correlate the metal content with the respiratory rate of a tissue have been suggestive, a strict parallel surely does not hold; and most of the iron present, for example, is at any one time catalytically inert (Warburg and Kubowitz, 1928).   Further, sudden changes in respiratory rate could not depend on similar changes in metal content.   One would expect in cells, therefore, secondary systems able to play upon the primary catalysts so as to maintain a constant activity or abruptly increase (and, possibly less abruptly, decrease) it.   These systems might have to do mainly with structural organization (as, *e.g.*, adrenalin frees diastase or glycogenase from absorption in the salivary or liver cell (Lesser, 1921, 1927); and changes in colloidal "carriers" result from many conditions (Willstätter and Rohdewald, 1934)) or might act more directly upon the catalytic agent.   In case of the latter possibility, cells would contain substances able to enhance or depress the oxidative catalyses induced by metals. Recent evidence suggests that urease, papain, and the glycolytic enzyme of muscle contain the sulfhydryl group and are reversibly inactivated by oxidizers, reactivated by reducers (Michaelis and Runnström (1934); see for other literature).   Further, a coenzyme, often complex, is required for most glycolyses and oxidations (Andersson, 1934), and a common component of these systems, adenylpyrophosphate (Lohmann, 1931), is itself formed and destroyed in a complicated metabolic sequence (Parnas, Ostern, and Mann, 1934).   These mechanisms all are capable of acting to regulate the metabolic flow of cells.   More particularly, Michaelis and Solomon (1931) have obtained respiration accelerators from tissues, and it seemed a reasonable expectation that tissue inhibitors for metal catalyses should also be present.   The presence of specific inhibitors is here reported.   Of especial interest is a copper inhibitor in liver.   Preliminary studies indicate that, in different samples, the concentration of this inhibitor runs parallel to the therapeutic value of the liver extract in pernicious anemia.   A rôle of copper in hemoglobin formation has long been known (Elvehjem, 1935).

Inhibitors might act by forming less active complexes with the

active metals and could be tested for, therefore, by observing a diminution of metal catalysis in their presence. Since the quantity of active metal in tissues is itself small, the secondary substances might be present in minute amounts. The catalytic system described in Paper I adapts itself admirably, however, to the investigation of minute quantities of active substances. The small thiol and metal concentrations used in our test system are favorable from this view-point. Appropriate blanks have, of course, been performed to allow for the metal content in the tissue preparations themselves. Some simple inhibitors were also tested before the examination of tissues was undertaken. The phosphates, discussed in Paper I, belong in part in such a class.

Methods

Technique—The methods employed for the measurement of rates of oxidation are described in detail in Paper I. There, also, is described the method used for expressing those rates. In the present experiments no fundamental changes in the procedure were required, and the extracts or other substances were added to the test solution as it was prepared with no untoward difficulty. The pH of the solution was adjusted in the usual manner, or when colored extracts precluded the colorimetric method, a hydrogen electrode was used.

To avoid weighing exceedingly small amounts of material, stock solutions, from which aliquots were drawn, were used in many cases.

Preparation of Extracts—To obtain reproducible results, certain precautions must be taken in handling the tissue extracts. The tissue must be extracted while fresh. At first tumor material was collected during a week (50 hens were inoculated for each batch) and kept frozen. The extracts, finally obtained, were then cloudy and gave atypical results. Presumably the freezing and thawing released additional material. The final extracts should be kept free of moisture and oxygen, within reasonable limits, and will then yield reproducible results for at least 4 months. After 6 months the results become unreliable.

The method outlined was employed in the preparation of the muscle, liver, and tumor extracts. The absolute, but not relative, amounts used may of course be varied.

200 gm. of tissue are ground into 60 cc. of H_2O and stirred for

half an hour.   295 cc. of 95 per cent ethyl alcohol are added, the mixture centrifuged, the residue resuspended in 115 cc. of the alcohol, stirred a short time, and again centrifuged.   The centrifugates are combined and the solvents removed under reduced pressure, during which the temperature is not allowed to exceed 35°. The residual material is dried *in vacuo* over phosphoric anhydride and powdered.   It weighs about 6 gm.

For the preparation of saline-extracted samples, tumor tissue is chopped coarsely into 50 cc. of 0.9 per cent sodium chloride solution, stirred for 2 to 3 minutes, and centrifuged; the residue is washed with 25 cc. of H_2O and again centrifuged.   The washings are discarded.   The residue is now treated as above.   The preliminary washing serves to remove extracellular débris.

Results

Simple Inhibitors—The catalytic action of copper is inhibited by methyl isocyanide (R—NC) about as powerfully as by the inorganic cyanides. 2×10^{-5} M KCN, for example, diminishes the action of 10^{-5} M Cu^{++} by 14 per cent; CH_3NC, by 8 per cent; and 10 times this concentration of the isocyanide gives over 98 per cent inhibition.   The isomeric methyl cyanide (R—CN), on the other hand, is ineffective even in 0.01 M concentration.   Tartaric acid is about one-fourth as powerful an inhibitor for copper as is the isocyanide—at molar ratios to copper of 10:1, the former causes an 11 per cent, the latter a 46 per cent decrease—a finding in harmony with the general relative stability of the two complexes formed.   Cupferron, forming a slightly soluble copper complex (Baudisch, 1909), gives an 8 per cent inhibition (molar ratio to Cu 20:1).   Glycine, in small concentration, has no effect, though it forms a complex inner salt with copper (Borsook and Thimann, 1932), and in large amounts, 0.3 M, gives an actual acceleration. Sodium dimethyl dithiocarbamate, containing non-oxidizable sulfur groups, forms a very stable copper complex (cherry-red) and inhibits thioglycolic acid oxidation as powerfully as does the isocyanide.

Since colloidal tissue preparations were to be examined, it was necessary to test the possible inhibitory action of colloids.   Gelatin $(3 \times 10^{-4}$ M) depresses copper activity by 14 per cent, does not affect iron, and increases that of manganese (owing, possibly, to a

copper impurity in it). Egg albumin, on the other hand, strongly inhibits copper and manganese (iron was not tested), the former

TABLE I

Effect of Various Substances on Rate of Oxidation of Thioglycolic Acid in Presence of Metals (Titrimetric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7; oxygen supplied at 4 liters per hour. Rate expressed in terms of (moles of R—SH oxidized $\times 10^6$)/(time in minutes).

Inhibitor	Concentration	Catalyst	Rate	Change in rate
	M	$M \times 10^5$		per cent
		0	0.7	
		1 Cu	122	
		0.5 "	49	
		4.0 Fe	36	
		0.5 Mn	77	
Potassium cyanide............	2×10^{-5}	1 Cu	105	−14
Methyl isocyanide............	2×10^{-5}	1 "	112	−8
" "	1×10^{-4}	1 "	56	−54
" "	2×10^{-4}	1 "	3	−98
" "	1×10^{-2}	1 "	0	−100
" cyanide..............	1×10^{-2}	1 "	119	−2
Tartaric acid.................	5×10^{-5}	0	4	
" "	5×10^{-5}	0.5 Cu	48	−10
Cupferron.....	5×10^{-5}	0	7	
"	5×10^{-5}	0.5 Cu	57	+2
"	1×10^{-4}	0.5 "	52	−8
Glycine......................	6×10^{-5}	1 "	119	−2
"	3×10^{-1}	0.5 "	80	+63
Sodium dimethyl dithiocarbamate....................	2×10^{-5}	1 "	104	−15
Potassium iodide...	4×10^{-2}	1 "	52	−57
Egg albumin	4×10^{-4}	1 "	38	−69
" "	4×10^{-4}	0.5 Mn	45	−42
Gelatin......................	3×10^{-4}	0	13	
"	3×10^{-4}	0.5 Cu	55	−14
"	3×10^{-4}	4 Fe	50	+3
"	3×10^{-4}	0.5 Mn	132	+55
		10 $K_4Fe(CN)_6$	4	

about as powerfully as the isocyanide. It seems to follow that colloids or proteins *per se* do not inhibit, though certain members of

the group may do so.   This point is reinforced by the findings with particular tissue extracts which exhibit specific and individual inhibitory effects.

Table I summarizes the effects of these substances on the heavy metal catalyses of thioglycolic acid oxidation, under varied conditions.   Each datum represents several experiments, none of which varied by more than ±5 per cent from the mean.   For purposes of comparison, the rates in the absence of inhibitors are placed at the head of the table.   It will be observed that in the absence of heavy metal ions, the rate of oxidation of thioglycolic acid is negligible.

Muscle—Beef or chicken muscle, ground in water and extracted with alcohol, yields finally a small amount of powder which is strongly inhibitory to iron.   The same material increases the activity of copper or manganese, possibly because of the presence in it of these metals and their consequent mutual acceleration (*cf.* Paper I).   Copper and iron added together sum their separate effects in the presence of the extract as well as in simple buffers; and since added iron exhibits only 40 to 50 per cent of its normal activity, the presence of a specific inhibitor for this ion is indicated (Table II).   The inhibitor could hardly be a phosphate in view of the activity of manganese, for in all cases manganese is more sensitive than iron to phosphate inhibition.

Note that the extract of chicken muscle inhibits the iron catalysis more than does a 4 times more concentrated extract of beef muscle.   The acceleration by extract alone, conversely, is distinctly less for chicken muscle, even at an equivalent concentration.

Sarcoma—Rous sarcoma, obtained by injection into the pectoral muscles of Plymouth Rock hens of the Rous powder (generously supplied us by Dr. Murphy of the Rockefeller Institute), behaved much like the muscle in which it developed.   Although the tumor tissue was meticulously separated from normal muscle, necrotic fragments, clotted blood, mucoid material, and the like, the final product must be regarded as composed of only relatively pure sarcoma cells.   When this material is extracted in the same manner as muscle, the iron inhibitor (as well as the copper or manganese accelerators) is again found, but the inhibitory effect is less intense than with muscle (Table III).   The inhibition observed is surely more than could be exerted by any contaminating

TABLE II

Inhibition by Muscle Extracts of Iron Catalysis of Thioglycolic Acid Oxidation

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. Muscle extract present in concentration of 11 gm. per liter of thioglycolic acid solution. (Refer to Table I for separate effects of metal ions.)

Muscle extract added	Catalyst	Rate $\frac{\text{Moles R—SH oxidized} \times 10^6}{\text{Time in min.}}$	Change in rate
Titrimetric measurements			
	$M \times 10^5$		*per cent*
Beef	0	27	
"	0.5 Cu^{++}	102	+54
"	4 Fe^{++}	45	−50
"	0.5 Mn^{++}	123	+25
"	0.5 Cu^{++}+ 4Fe^{++}	122*	
Manometric measurements			
		O₂ absorbed per 100 min.	
		c.mm.	
	10 Fe^{++}	90	
Beef	0	48	
"	10 "	91	−52

Manometric measurements. Experimental constants, as above, except concentration of extract is smaller, 2.5 gm. per liter of thioglycolic acid solution.

	0.5 Cu^{++}	45	
	10.0 Fe^{++}	90	
	0.5 Mn^{++}	55	
Chicken		8	
"	0.5 Cu^{++}	64	+45
"	10 0 Fe^{++}	43	−61
"	0.5 Mn^{++}	95	+58

* Copper ions do not affect iron catalysis. Thus:

Rate for extract and copper ions.................................. 102

"   "   iron in presence of extract (50% of 36)..................... 18

Total.. 120

Compare with the observed value of 122.

muscle present, and we conclude that the iron inhibitor is present in reduced amount in the cancer cells. Consistent results were obtained with six separate lots of sarcoma. It is suggestive that

in cancer, which is characterized by a loss of controlled or restrained growth, by increased glycolysis, and the like, there is a decrease in an inhibitory agent for iron, as compared to its tissue of origin.   This is the more interesting since iron catalysts are

TABLE III

Effect of Rous Sarcoma Extracts* on the Rate of Metal-Catalyzed Thiol Oxidation (Manometric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. Concentration of extracts 2.5 gm. per liter of solution.

Substance added				Catalyst	O_2 absorbed per 100 min.	Change in rate
				$M \times 10^5$	*c.mm.*	*per cent*
None				0.5 Cu^{++}	45	
"				10.0 Fe^{++}	90	
"				0.5 Mn^{++}	55	
Rous sarcoma, Extract A					17	
"	"	"	"	0.5 Cu^{++}	72	$+22$
"	"	"	"	10.0 Fe^{++}	92	-17
"	"	"	"	0.5 Mn^{++}	138	$+120$
"	"	"	1-B		3	
"	"	"	1-B	0.5 Cu^{++}	96	$+107$
"	"	"	1-B	10.0 Fe^{++}	65	-31
"	"	"	1-B	0.5 Mn^{++}	129	$+129$
"	"	"	2-B		11	
"	"	"	2-B	0.5 Cu^{++}	89	$+73$
"	"	"	2-B	10.0 Fe^{++}	34	-71
"	"	"	2-B	0.5 Mn^{++}	22	-80

* Extract A is the standard preparation; Extract 1-B, the one after saline washing of the tumor.   Extract 1-B probably contains less metal, since it induces a lower rate of oxidation of thioglycolic acid.   The sarcoma Extract 2-B was prepared as for Extract 1-B from tissue kept frozen over a week.   In this case manganese catalysis is inhibited, whereas extracts prepared from fresh tumor always accelerate.   The inhibition may well be due to inorganic phosphates produced by the hydrolysis of organic phosphates.   In harmony with this possibility, iron catalysis is inhibited also, but to a lesser degree than manganese (see Paper I).

apparently involved in glycolysis (non-hemin Fe^{++}) (Zuckerkandl, Fleischmann, and Drucker, 1934) as well as in several oxidation systems.

The catalytic action of the tumor extract without added metals is high, more than that of muscle, which suggests a large metal

content. This is probably due to necrotic tissue and blood not fully removed from the sarcoma. As evidence for this, if the fresh tissue is coarsely chopped and washed once with isotonic saline solution before the usual extraction, its intrinsic catalytic action is largely lost, while its other effects are unaltered. Since this washing should remove soluble material not held by the semi-permeable membranes of living cells, the bulk of the catalytic effect of the extract is presumably derived from extracellular material, the specific inhibitor from the living cells. Control experiments on blood (see Table VI) further support the view that

TABLE IV

Effect of Liver Extracts on the Metal Catalysis of Thiol Oxidation (Mano-metric Measurements)

* Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8 7; 2.5 gm. of liver extract per liter of solution.

Substance added	Catalyst	O_2 absorbed per 100 min.	Change in rate
	$M \times 10^5$	*c.mm.*	*per cent*
	0.5 Cu^{++}	45	
	10.0 Fe^{++}	80	
	0.5 Mn^{++}	55	
Chicken liver, Extract A		15	
" " " "	0.5 Cu^{++}	42	−40
" " " "	10.0 Fe^{++}	96	+1
" " " "	0.5 Mn^{++}	148	+142

specific cellular substances are involved in the inhibitory action of tissues.

Liver—Chicken liver was extracted by means of the identical procedure applied to the other materials, yet the liver extract affected the metal catalyses in an entirely different manner. This fact greatly enhances the probability that specific substances, rather than some overlooked feature of the general conditions, are responsible for the findings. Liver extract is entirely neutral to iron and inhibits copper, while manganese, as in the other cases, is accelerated (Table IV).

Liver extracts prepared in a variety of ways exhibit a wide variation in their effectiveness as copper inhibitors (we are indebted to Eli Lilly and Company for supplying us with a

TABLE V

Effect of Liver Extracts on Metal Catalysis of Thiol Oxidation (Titrimetric Measurements)

Temperature 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. The extract was present in concentration of 11 gm. per liter of solution.

Extract No.	O₂ absorbed per 100 min. (no metal added)	Catalyst	O₂ absorbed per 100 min. (with catalyst)	Change in rate	Clinical results
	c.mm.	M × 10⁵	c.mm.	per cent	
		0.5 Cu⁺⁺	49		
		1.0 "	122		
		2.0 "	270		
		4.0 Fe⁺⁺	35		
		0.5 Mn⁺⁺	77		
Liver					
841015	11	0.5 Cu⁺⁺	45	−31	+
841015 (digested with concentrated HCl)	134	0.5 "	196	+27	
841015	11	4.0 Fe⁺⁺	31	−43	
841015	11	0.5 Mn⁺⁺	97	+12	
E-191	10	0.5 Cu⁺⁺	41	−37	
E-197	13	0.5 "	17	−92	
E-197	13	1.0 "	22	−93	
E-197	13	2.0 "	49	−87	
E-330-B	46	0.5 "	95	0	
E-352	15	0.5 "	25	−80	+
E-352	15	0.5 Mn⁺⁺	148	+73	
E-353	20	0.5 Cu⁺⁺	87	+37	
E-374-A	28	0.5 "	97	+41	−
E-374-B	34	0.5 "	41	−86	
E-375	49	0.5 "	84	−29	
E-377-A	17	0.5 "	46	−41	
E-377-B	81	0.5 "	136	+12	− ·
E-379-A	31	0.5 "	87	+14	−
Kidney					
E-196	78	0.5 "	186	+120	
Spleen					
E-198	57	0.5 "	118	+24	

number of such extracts).[1] These extracts were prepared in connection with the therapy of pernicious anemia and several had been assayed for therapeutic action in the Lilly laboratories by means of their usual clinical tests.   We found, on comparing our assay of the inhibitory action of these several extracts on copper catalysis with such of their results on therapeutic efficacy as were available, that the two properties ran parallel.   Liver preparations that failed to inhibit copper also failed to improve pernicious anemia patients; those that did inhibit copper improved patients. A few experiments with spleen and kidney extracts prepared along similar lines failed to demonstrate anti-catalytic activity.   Some results are collected in Table V.

TABLE VI

Effect of Blood Extract on Metal Catalysis of Thiol Oxidation (Manometric Measurements)

The experimental constants were the same as for Table III, except that 0.5 gm. of extract per liter of solution was used.

Substance added	Catalyst	O$_2$ absorbed per 100 min.	Change in rate
	$M \times 10^5$	*c.mm.*	*per cent*
	0.5 Cu^{++}	45	
	10.0 Fe^{++}	90	
	0.5 Mn^{++}	55	
Blood extract		9	
" "	0.5 Cu^{++}	68	+29
" "	10.0 Fe^{++}	117	+17
" "	0.5 Mn^{++}	192	+232

This parallelism, of course, suggests the availability of the catalytic method for assaying the clinical value of liver extracts, and the experiments so far conducted warrant further study and a more complete separate report.   Further, these results again emphasize the specific identity of catalytic inhibitors (and, less surely, accelerators) present in the various body cells.

Blood—Fresh blood was extracted by the usual method, 1500 gm. of sheep blood yielding 8 gm. of extract.   Its effect on heavy metal catalyses is illustrated in Table VI.

[1] Some of these extracts showed also a strong inhibitory effect upon iron. This effect could be directly traced to an extraction procedure which allowed the introduction of specific iron inhibitors.

The effects of the blood extract on copper, iron, and manganese ion catalyses are accelerations of 29, 17, and 232 per cent respectively. It is possible from these data to estimate the influence of blood contained in the sarcoma tissues investigated. Assuming that 10 per cent of the fresh weight is blood (surely a generous figure), 20 gm. of blood would be extracted in the usual 200 gm. sample. This blood would yield 0.1 gm. of extract to 5 gm. for the whole sample, or 2 per cent. 5 mg. of the whole, used in a test, would contain 0.1 mg. of blood extract, which, by extrapolation from the above results with 1 mg., would accelerate copper 4 per cent, iron 2 per cent, and manganese about 20 per cent. Except for the last, such effects are negligible and, since all are in a positive direction, they could not account for the specific inhibitory effect of sarcoma extracts.

SUMMARY

1. The ability of tissue extracts as well as of a group of specific substances to enhance or decrease the rate of metal-catalyzed oxidation of thioglycolic acid was studied by the methods described in Paper I.

2. A number of chemicals able to form complexes with copper ion inhibit its catalytic action to an extent roughly paralleling the stability of the complex. Colloidal materials, as such, are inactive.

3. Beef or chicken muscle yields extracts highly inhibitory to iron, somewhat acceleratory to copper and manganese.

4. Chicken sarcoma (Rous) extracts act like muscle, but are less powerful.

5. Chicken liver extracts are neutral to iron, accelerate manganese, and inhibit copper. In a few cases where direct comparison was made, the intensity of copper inhibition produced by various extracts of liver paralleled their therapeutic value in the treatment of pernicious anemia.

6. Control tests with blood extracts exclude blood as a factor in the above results.

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THE OXIDATION OF CYSTINE IN NON-AQUEOUS MEDIA

V. ISOLATION OF A DISULFOXIDE OF l-CYSTINE*

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With a Rational Method for Its Production by Theodore F. Lavine

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Preceding studies on the oxidation of cystine perchlorate in acetonitrile by means of perbenzoic acid made it evident that prior to the complete oxygenation of the —S—S— group an intermediate compound or compounds are formed that are reducible to cystine by iodide in acid solution (1). The isolation[1] of a cystine dioxide which possesses this property is described in the present paper. The disulfoxide (R—SO—SO—R) structure rather than the thiolsulfonate or monosulfone (R—S—SO₂—R) form is suggested for the new compound on the basis of its easy reduction to cystine.

The disulfoxide was separated from other reaction products by virtue of its slight solubility and relative stability in water. Since these properties make a separation from cystine practically impossible, it was imperative that no unoxidized cystine should remain at the end of the oxidation. Accordingly, oxidation was allowed to proceed in the solution of cystine perchlorate and perbenzoic acid until the cystine test had become negative; the mixture was then neutralized with pyridine, forming a crude precipitate of the cystine reaction products which after sparing extraction with water yielded the disulfoxide as a white powder.

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[1] Preliminary reports have been made previously (2).

The yield was found to be dependent on the extent of formation of oxidation products higher than the disulfoxide at the time of precipitation. Systematic study of the effect of variations in the conditions of oxidation showed that increased temperature lessened the overlapping of the different oxidation steps by favoring the speed of formation of the disulfoxide from cystine in comparison with the speed of oxidation of the disulfoxide to higher products. Thus by carrying out the oxidation at 25° (instead of −10°) and using a slight excess over the theoretical amount of perbenzoic acid, disulfoxide preparations of 99.5 per cent purity could be obtained in 75 per cent yield.

That the compound represents an oxidation stage corresponding to the disulfoxide was determined by quantitative oxidation to cysteic acid by excess I_2 in dilute acid solution, and by quantitative reduction to cystine by KI-HCl; the I_2 consumed in the first case and that liberated in the second corresponding with requirements for the indicated changes of the disulfoxide.

$$R—(SO)_2—R + 3I_2 + 4H_2O = 2R—SO_3H + 6HI$$

and

$$R—(SO)_2—R + 4HI = R—S—S—R + 2I_2 + 2H_2O$$
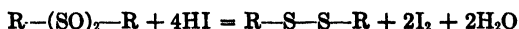
The *l*-cystine regenerated by the reduction was almost quantitatively recovered.

The amphoteric properties of cystine are still exhibited by the disulfoxide, although its isoelectric range is shortened and its isoelectric point shifted distinctly toward the acid side (pH 3). This shift appears of interest as an extension to an ampholyte of observations made by various authors (3–5), showing that in sulfur-containing carboxylic acids, the oxidation of sulfide to sulfoxide and sulfone increases the acidity. The isoelectric solubility of the disulfoxide is greater than that of cystine, while its optical rotation, although still negative, is smaller.

One of us (T.F.L.) wishes to acknowledge here his appreciation to Professor E. C. Wagner of the University of Pennsylvania for his continued interest in the course of this work.

EXPERIMENTAL

The method of preparing anhydrous acetonitrile solutions of cystine perchlorate has been previously described (6). Per-

benzoic acid was used in either chloroform or acetonitrile solution
(1) without apparent difference in results. The various tests
employed in the earlier work (1) were further investigated and
improved as follows.

Determination of Oxygen Consumption—The extent to which
oxidation had proceeded in the non-aqueous solution was deter-
mined by titration of the I_2 liberated from slightly acidified
(0.05 N H_2SO_4) N KI by the action of the unused perbenzoic acid
in aliquots of the oxidation solution. The results, however, were
found to be in error owing to the oxidizing action of the partially
oxidized cystine, for, removal of unused perbenzoic acid from
aliquots (1 or 2 cc.) after addition to 0.1 N HCl (10 cc.), by seven
rapid extractions with $CHCl_3$ (1 or 2 cc.), and titration of the I_2
liberated by the combined $CHCl_3$ extracts yielded results which
were 1.16 ± 0.04 times the direct titration (calculated as gm.
atoms of oxygen consumed per mole of cystine). This correction
factor has been applied to all direct titrations although it may be
erroneous in the very early stages of oxidation. Owing to con-
tinuing oxidation during the extraction, there occurs a loss in
perbenzoic acid (and cystine), which was found to decrease as the
ratio of perbenzoic acid to cystine diminishes (being 7 per cent at
a ratio of 2:1). The low ratios of added O to —S—S— used in
most of the following oxidations would probably make losses from
this source still smaller, especially near the end of the oxidation.

Colorimetric Determination of Cystine and Intermediates—Cys-
tine and intermediates reducible by HCl and KI to cystine were
determined by application of the cyanide-nitroprusside test to
the above aqueous HCl extract (1) and the course of the non-
aqueous oxidation was interpreted from these results, although
dismutative rearrangements might occur in the aqueous solution.
Since the intensity of color developed in the nitroprusside test
depends in part on the NH_4Cl present and the alkalinity, the test
for cystine was modified slightly by increasing the HCl content of
test and standard solutions to 1.5 ± 0.2 mM per 4 cc., so that the
same standards could be used for the two colorimetric determi-
nations which were run simultaneously. It was found that solu-
tions of the disulfoxide do not respond to this test even after
40 minutes standing in the ammoniacal cyanide solution. In the
estimation of the intermediate oxidation products, 3 drops of 10

N HCl and 4 drops of saturated KI were added to a volume of the solution to be tested, preferably less than 1 cc.; after standing for 20 to 30 minutes in darkness or diffuse daylight, the I_2 liberated by the reduction was removed by adding 1 cc. of 8.6 N NH_3 and stirring; the solutions were then diluted to 4 cc. with H_2O, treated with NaCN and sodium nitroprusside, as in the test for cystine, and the amount of cystine formed was estimated by comparison with the cystine standards. Deduction of the cystine found in the first test from this last result yielded the amount of intermediate present.

Results with an accuracy of ±5 per cent have been obtained with solutions of the pure disulfoxide, but results on the above mentioned aqueous extracts showed greater fluctuations. This may be partially due to the fact that there are other intermediate compounds besides the disulfoxide which are capable of reduction to cystine by the action of KI-HCl. In fact, the sulfinic acid (R—SO_2H), as prepared by Schubert (7), and the sulfenic acid (R—SOH) (8) were found to respond to this test. However, application to the disulfone (R—$(SO_2)_2$—R) (9) showed this compound to either be non-reducible or to yield on hydrolysis an isomeric sulfinic acid which resists reduction under these conditions.

Oxidation Level of Reaction Products—The degree of oxidation of isolated materials was determined by the iodine (oxygen) consumed on complete oxidation by excess iodine (at least 100 per cent) in slightly acid (0.04 N HCl) solution. Since it has been shown (10) that under these conditions cystine is oxidized to cysteic acid, this compound may be assumed to represent the end-product of this oxidation, especially since no appreciable sulfate formation occurs with either cystine or the disulfoxide. Results obtained by this method are reported in Table I, Columns 12, 16, and 20, as the amount of material (gm.) oxidized by 1 gm. atom of oxygen; *i.e.*, 1 gm. atom of oxygen would oxidize 90.7 gm. of disulfoxide (272:3) or 48 gm. of cystine (240:5), etc., to cysteic acid. The oxidations were run in each case with a blank and continued until constant values were obtained (usually overnight).

Oxidations and Isolation of Disulfoxide—All the following oxidations were allowed to proceed until the colorimetric test for cystine

became practically negative (usually somewhat longer because of the time necessary for running the colorimetric tests, etc.). Any precipitate of higher oxidation products (9) present at this point was generally filtered off, although it was subsequently found that owing to its water solubility the amounts produced do not interfere with the isolation of the disulfoxide and therefore need not be removed. The cystine reaction products remaining in solution are precipitated by the addition of a slight excess of pyridine
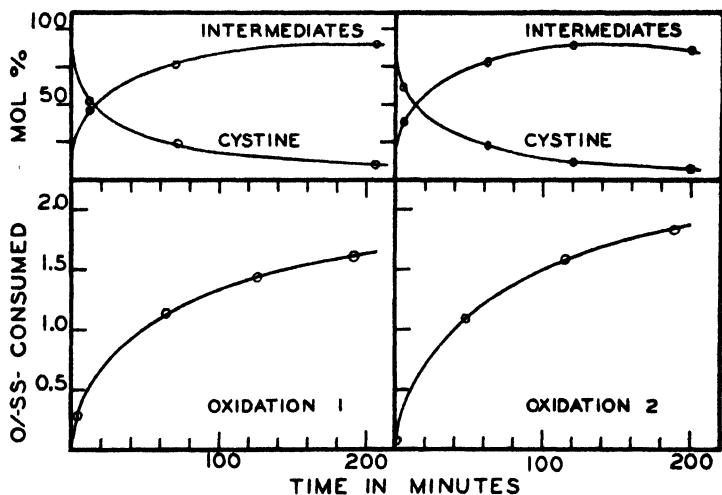
Fig. 1. Oxidation of cystine perchlorate in CH₃CN at −10°. Oxidation 1 represents cystine perchlorate 0.025 M, perbenzoic acid 0.0525 M; Oxidation 2, cystine perchlorate 0.025 M, perbenzoic acid 0.075 M. Cystine and intermediates are expressed as molar percentages of the original cystine taken.

(calculated according to the perchloric acid present); the gelatinous nature of this precipitate makes filtration and washing (with CH₃CN) difficult and tedious, especially with large amounts. On drying *in vacuo* at room temperature formation of SO₂ was sometimes noted, indicating decomposition which possibly accounts for the small amounts of cystine found in the dried material. The disulfoxide was isolated by digesting this precipitate with water (3 to 4 cc. per gm.), which formed on standing about 10 minutes a more or less pasty mass varying in color from

yellow to brown in different preparations. The mixture was filtered by suction and the residue washed with water until the washings were colorless, then with alcohol and ether, and finally dried *in vacuo* at room temperature over P_2O_5 or $CaCl_2$.

The course of two oxidations carried out at $-10°$ are shown in Fig. 1, where oxygen consumption is plotted together with colorimetric determinations of cystine and intermediates. Occasionally, owing to unexplained fluctuations in the KI-nitroprusside test, values were obtained for total cystine and intermediates which were greater than the original cystine content; in such cases the theoretical total was used for calculation of the intermediates present.

The curves suggest, in agreement with observations made in another study (11), that the monosulfoxide of cystine (R—SO—S—R) does not respond to the cyanide-nitroprusside test for cystine, since in both oxidations of Fig. 1 the cystine became 50 per cent when 0.5 atom of O per —S—S— was consumed, or when 0.5 mole of R—SO—S—R was formed from 1 mole of R—S—S—R. The data obtained in working up the products of these two oxidations are given in Table I (Preparations 1 and 2). The unsatisfactory results (formation of considerable amounts of higher oxidation products, low yields, and a final product contaminated with cystine) prompted the other experiments recorded in Table I in which the perbenzoic acid to cystine ratio, total concentration, and temperature were varied. Some of the colorimetric determinations of intermediates may be low owing to insufficient time of reduction. The precipitate of higher oxidation products, Precipitate A, which was filtered off and analyzed where shown, displayed wide variations in composition, which may be largely due to occlusion caused by its densely granular structure or to its hygroscopic nature (*cf.* (9)). However, the amount of this precipitate decreased as the temperature (and initial perbenzoic acid to cystine ratio) was increased, until at room temperature only a negligible turbidity was produced at the stage of oxidation corresponding to the consumption of 2 oxygen atoms per mole of cystine. Comparisons of the yields of disulfoxide in Preparations 4 and 5 show that the presence of this precipitate does not in itself affect the final results. Preparations 8 to 10 (0.6 to 1.2 gm. of cystine were used) show the

TABLE I

Summary of Non-Aqueous Oxidations of Cystine Perchlorate and Isolation of Cystine Disulfoxide

Cystine and intermediates were determined colorimetrically; the latter were calculated as disulfoxide.

(1) Preparation No.	(2) Temperature °C.	(3) Cystine perchlorate M	(4) Ratio O to —S—S— added	Oxidation (5) Time for consumption of 2O per —S—S— hrs.	(6) Total time of oxidation hrs.	(7) Total O consumed per mole —S—S— gm. atoms	(8) Sulfur present as intermediates (disulfoxide) per cent	Precipitate A (higher oxidation products) (9) Yield* per cent	(10) Amount of cystine present per cent	(11) Amount of intermediate (disulfoxide) present per cent	(12) Amount oxidized to R—SO_3H by 1 gm. atom oxygen gm.	Precipitate B (by pyridine) (13) Yield* per cent	(14) Amount of cystine present per cent	(15) Amount of intermediates (disulfoxide) present per cent	(16) Amount oxidized to R—SO_3H by 1 gm. atom oxygen gm.	Isolation of disulfoxide (17) Amount of B treated with 1 cc. H₂O gm.	(18) Yield* per cent	(19) Amount of cystine present per cent	(20) Amount oxidized to R—SO_3H by 1 gm. atom oxygen gm.
1	−10	0.025	2.1		20	2.06	73	22	0.3	4	932	72	4.8	50	106	0.28	32	25	87.4
2	−10	0.025	3	4	24	2.9	64	14	1.3	13	608	85	2.7	58	165	0.24	14	2	90.7
3	−10	0.025	6		2	2.1	75	15	11.5	12.1	228	85	1.4	50	130	0.25	36	2	91
4	0	0.05	3	0.83	4.86	2.4	85	8	3.7	22	233	92	1.9	72	108	0.28	60	<0.7	91.5
5	0	0.05	3	0.83	2.8	2.4	95	4	Not removed			100	2.2	71	103	0.34	50	<0.9	90.5
6	0	0.05	4	0.38	1.2	2.5	65	4	2.6	15	331	96	2.6	61	117	0.33	50	4.3	84.9
7	0	0.05	5	0.22	0.53	2.4		5	5.5	25	224	95	0.4	60	107	0.29	48	11	84.8
8	20	0.05	2.4	0.27	1.9	2.16	56	Not removed	“	“	“	100	2.2	69	96.5	0.31	73	<0.7	90.7
9	24	0.075	2.4	0.125	0.67	2.12	80	Not removed	“	“	“	100	3	77	94	0.31	76	<0.5	90.4
10†	25	0.05	2.4	0.27	1.1	2.18	65	Not removed	“	“	“	100	1.8	84	94	0.34	73	<0.5	91.0

* The yields are calculated on the basis of the sum of the cystine used and the total oxygen consumed.

† The solution contained 15 per cent by volume of chloroform.

most favorable conditions for the production of a pure disulfoxide in good yield.

Physical Properties and Analysis—The disulfoxide is obtained by the above method of isolation as a white powder.   Dilution of a saturated solution in N HCl to 0.1 N yielded the crystals shown in Fig. 2.

The compound melts with decomposition and foaming, after first sintering, at 179–182° (corrected).   It is not hygroscopic and is apparently stable in the dry state.   Drying at room temperature *in vacuo* over P_2O_5 appears safest, since SO_2 was noticed occasionally even at 56°, accompanied by a slight increase (0.5 per cent) in the cystine content, as indicated by the cyanide-nitroprusside test.

The solution of the disulfoxide in N HCl (1 gm. per 100 cc.) shows an initial optical rotation of $[\alpha]_{Hg}^{29} = -30.2°$.   This value, however, becomes increasingly negative owing to decomposition, which will be treated in a succeeding paper.

Different preparations gave the following analytical values.

$C_6H_{12}O_4N_2S_2$ (272.24)
Calculated.   C 26.45, H 4.445, S 23.56, N 10.29
Found.   " 26.2,   " 4.75,   " 23.33, " 10.20 (macro-Dumas)
                   " 26.8,   " 4.55,   " 23.49, " 10.42   "
                   " 26.2,   " 4.59,   " 23.35, " 10.8 (Van Slyke, with factor
                                                                            0.926)
C and H values are averages of duplicate microdeterminations.

Oxidation to Cysteic Acid and Reduction to Cystine—The disulfoxide has been shown to consume an amount of iodine corresponding to oxidation to cysteic acid (*cf.* Table I).   Quantitative reduction to cystine was demonstrated as follows: 0.1133 gm. of the disulfoxide was allowed to stand for about an hour with 1.17 cc. of 10 N HCl and 2 cc. of 5 N KI; after dilution to 10 cc. with H_2O, the I_2 developed was extracted with ether and titrated with $Na_2S_2O_3$.   96.5 per cent of the theoretical amount was found. An equivalent amount of cystine (0.0998 gm.) was treated in the same way (with 1.0 cc. of 10 N HCl and 2 cc. of 5 N KI) and used as a control.   The optical rotations (per dm.) of the two aqueous extracts showed 99 per cent agreement (*i.e.* −2.12°, −2.14°). The cystine content of the reduced solution, calculated from the iodine consumption in oxidation to cysteic acid by an excess of
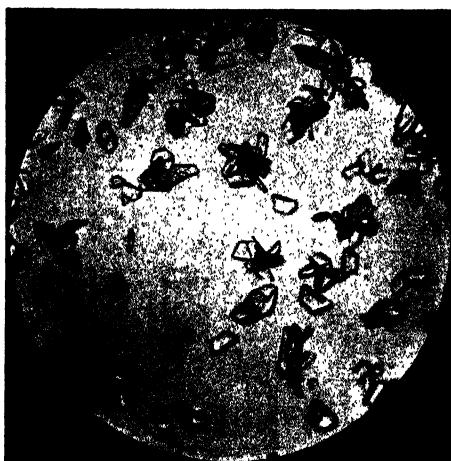
Fig. 2. Crystals of cystine disulfoxide × 41.5
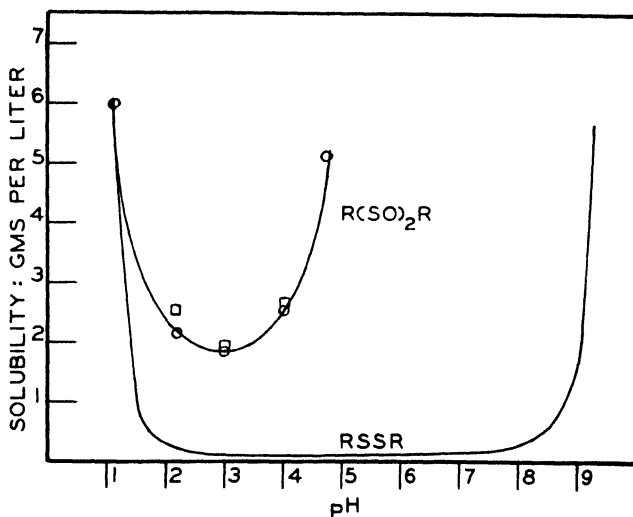
Fig. 3. Solubility of cystine disulfoxide compared with that of cystine (data of Sano (13)). The squares represent results from supersaturated solutions; the circles, results from shaking unsaturated solutions with an excess of substance.

I_2, was 97 per cent of the control. Neutralization of another aliquot of the reduced solution to pH 6 yielded the hexagonal

plates of *l*-cystine in a practically quantitative yield (7 per cent remained in solution), identified by the nitroprusside test and optical rotation.

Solubility—The compound was found to be amphoteric with its isoelectric point at pH 3, as determined by visual comparison of the amounts of precipitate in progressively neutralized 0.1 N HCl solutions and by solubility determinations at different hydrogen ion concentrations. Fig. 3 shows data obtained on the one hand by shaking buffered solutions (Clark's 0.05 M buffers (12)) with an excess of disulfoxide for 24 hours at room temperature (approximately 25°), and on the other by allowing supersaturated solutions (dissolved in HCl, neutralized, and buffered) to stand for 24 hours. The amount of disulfoxide in solution was determined from oxidation by I_2 (*cf.* under oxidation level of reaction products). The accuracy of these determinations is limited by the instability of the disulfoxide in solution, which becomes more pronounced with increasing pH.

An Alternative Method of Production

By Theodore F. Lavine

The following procedure for the preparation of cystine disulfoxide embodies certain simplifying changes designed to eliminate that step in the preparation involving the precipitation of the reaction products by pyridine and the attendant difficulties in the subsequent handling of this precipitate, with the result that now the amount which can be conveniently prepared depends only on the facilities available. The modifications introduced are based on the relative stability of the disulfoxide in aqueous acid solution and on the fact that acetonitrile-chloroform mixtures are immiscible with water.

12.01 gm. of cystine (0.05 mole) are transferred to a liter flask with about 250 cc. of acetonitrile (a volumetric flask is used when determinations of oxygen consumption, etc., are to be made). To the suspension there is added with good cooling and shaking, first a weighed amount of accurately standardized 70 per cent perchloric acid, exactly equivalent to the cystine (0.10 mole of $HClO_4$), together with 300 cc. of acetonitrile, and then a volu-

metrically measured amount of acetic anhydride, equimolar to 90 to 95 per cent of the water introduced with the perchloric acid. The mixture is shaken until the cystine has dissolved, filtered if necessary, and the solution allowed to come to room temperature. Then 0.12 mole of perbenzoic acid in about 150 cc. of chloroform[2] is added with shaking and slight cooling, and the solution made up to a liter with acetonitrile. After standing for an hour at 20–25°, the solution is transferred to a separatory funnel and extracted with 250 cc. of N HCl.[3] The lower aqueous layer is drained off and the oxidation solution reextracted with 100 cc. of N HCl. The two aqueous solutions are combined and extracted four or five times with 50 cc. portions of $CHCl_3$, until the volume of the drained off portion of $CHCl_3$ does not increase (due to removal of the CH_3CN taken up by the aqueous solution). After filtration the aqueous layer is slowly neutralized, with active stirring to prevent local excesses, with about 8 N NH_3,[4] until precipitation starts, the neutralization being completed with dilute NH_3 (1.0 N) to the beginning turning point of Congo red (paper). After standing about 10 minutes, the precipitate is filtered off by suction, washed with water, alcohol, and ether, and dried *in vacuo* at room temperature.

The yields of isolated disulfoxide from oxidations carried out as described were as follows: 72 per cent of the theoretical from oxidation of an anhydrous solution, 73 per cent from a solution approximately 0.013 M in H_2O, and 54 per cent from a solution 0.25 M in H_2O (no acetic anhydride added). These results indicate that although the stability of the disulfoxide makes the use of strictly anhydrous solutions unnecessary in the oxidation,

[3] Under the conditions of the subsequent extraction with N HCl, it was found that phase inversion of the system, $CHCl_3$-CH_3CN-N HCl, occurs between volume ratios of the constituents of 2:8:2.5 (where the N HCl is the lower layer) and 3:7:2.5 (where the N HCl is the upper layer). Furthermore, the separation of the lower aqueous layer was more rapid when the $CHCl_3$ content of the $CHCl_3$-CH_3CN solution was decreased from 20 to 10 per cent by volume.

[3] Precipitation of the disulfoxide occurred when less than 200 cc. of N HCl was used; the solubility of the disulfoxide in N HCl is about 6 gm. per 100 cc.

[4] Ammonia was used for neutralizing, since the danger of decomposition of the disulfoxide in contact with local excesses is less with ammonia than with the caustic alkalies, *cf.* Paper VI.

nevertheless it is advisable, for a good yield, to remove most of the water introduced with the 70 per cent perchloric acid.

Thanks are expressed to Merck and Company, Inc., Rahway, New Jersey, for generous supplies of cystine and acetonitrile and for the performance of the microanalyses.

SUMMARY

A new intermediate oxidation product of *l*-cystine, the disulfoxide, has been prepared by means of the non-aqueous oxidation of cystine perchlorate in acetonitrile with perbenzoic acid. The compound was identified by its analytical properties and level of oxidation.

An alternative method of isolation, facilitating preparation of the compound on a larger scale, is also described.

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THE OXIDATION OF CYSTINE IN NON-AQUEOUS MEDIA

VI. A STUDY OF THE REACTIONS OF THE DISULFOXIDE OF *l*-CYSTINE, ESPECIALLY OF ITS DISMUTATIVE DECOMPOSITIONS*

By THEODORE F. LAVINE

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The preparation and some of the properties of the disulfoxide of *l*-cystine have been described in a previous paper (1). Despite its intermediate level of oxidation, which is suggestive of chemical instability, the disulfoxide was shown to be relatively stable, especially in aqueous acid solution, a property which was utilized in its separation from other products of non-aqueous oxidation. However, further study revealed that all aqueous solutions of the disulfoxide undergo dismutative decomposition, or hydrolytic oxidation and reduction, which results in the formation of cystine and acid derivatives. The rate of this decomposition increases with increasing pH (and temperature); in N HCl 15 days are required for completion of the reaction, while slightly more than 15 minutes are sufficient at pH 8. Apparently the acidity of the solution also affects the course of the reaction (possibly through an isomerism of the disulfoxide), since analytical evidence indicates the acid derivative formed by alkaline decomposition to be the sulfinic acid (R—$S(O)$—OH), while the evidence from acid decomposition points to the formation of several acids. The sulfinic acid has been isolated and identified; it possesses some differences from that described by Schubert (2).

The chemical reactivity of the disulfoxide is illustrated by its rapid reaction with thiol compounds and NaCN. The sulfinic acid seems to be a product common to all these reactions.

The study of the decompositions and reactions of the disulfoxide was aided by the development of a quantitative method for the estimation of "intermediate oxygen," based on the oxidizing action of the disulfoxide, sulfinic acid, etc., on KI-HCl solutions.

Biologically, the relative stability of the disulfoxide, and more especially of the sulfinic acid, allows these compounds the more than transitory existence in the body necessary for their postulated rôles in biological redox systems in general and particularly in that involving the sulfur compounds (3).

EXPERIMENTAL

Determination of Intermediate Oxygen or Oxidizing Value—The I_2 liberated in the reduction of the disulfoxide to cystine by the action of KI and HCl (1) according to the equation

$$R—(SO)_x—R + 4HI \rightarrow R—S—S—R + 2I_2 + 2H_2O \qquad (1)$$
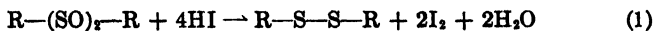
can be titrated directly with thiosulfate, thus furnishing a convenient method for the estimation of the intermediate oxygen attached to the cystine (or cysteine) sulfur. Results on the disulfoxide and on two preparations of sulfinic acid are given in Table I; the reduction of the sulfinic acid also leads to the formation of cystine ($2R—SO_2H + 6HI \rightarrow R—S—S—R + 3I_2 + 4H_2O$).

The indicated concentrations of HCl and KI were obtained by using 10 M HCl and 5 M KI as stock solutions; the latter remains colorless on storage at about 2°. The test solutions and corresponding blanks were allowed to stand in darkness in order to minimize the photochemical decomposition of the KI-HCl mixtures. Aliquots were withdrawn at the stated time intervals and the I_2 developed titrated with thiosulfate; if necessary the aliquots (of the blanks especially) were diluted with water so that the acid concentration was about 0.5 M at the end of the titration. The disappearance of the yellow I_2 color is most suitable as an end-point, although starch can be used, if the solution is shaken for about 10 seconds between each addition of thiosulfate (0.05 cc.), near the end-point (*cf.* (4)). The I_2 liberated by the blank was deducted from that formed in the test solution. Constant values were obtained after 1 hour, which persisted for about 4 hours, when concentrations of KI-HCl of 1 M or higher were used;

beyond this time there was a slow increase in the I_2 liberated, presumably due to different rates of decomposition of HI (KI-HCl) in the test and blank solutions.

That the reduction under these conditions proceeds only to cystine was established not only by the isolation of cystine after reduction (1), but also by the fact that cystine when dissolved in

TABLE I

I_2 Liberated by Intermediate Oxidation Products of Cystine (and Cysteine) on Treatment with KI-HCl *

The solutions contained approximately 0.1 gm. of intermediate per 50 cc.; 10 cc. aliquots were titrated with 0.025 N $Na_2S_2O_3$.

Compound	HCl	KI	Per cent of theoretical I_2 liberated after			
			1 hr.	2 hrs.	3 hrs.	4 hrs.
	M	M				
Cystine disulfoxide...............	0.5	0.5	46	56	62	†
" "	1.0	1.0	89.4	99.4	100.6	100.6
" "	2.0	2.0	98.6	100.3	100.3	100.6
Sulfinic acid‡....................	1.0	1.0	101	108	108	
" "	2.0	2.0	105	107	107	
" " §....................	1.5	1.5	100.0	100.6	100.8	101.2

* These experiments were conducted in a dark room (except for the actual titration); the reduction was somewhat faster when carried out in diffuse daylight (*i.e.*, in a dark corner or in a cabinet). *Cf.* foot-note §.

† The reduction was 80 per cent of the theoretical at 20 hours, but *decreased* from then on.

‡ This sulfinic acid was prepared according to Schubert (2) and possessed a molecular weight of 165, based on oxidation by an excess of I_2 (1); theory for $R—SO_2H$ is 153 and for $R—SO_2H·H_2O$, 171. If 153 is used, the values obtained would be 7.3 per cent lower; *i.e.*, practically the theoretical. However, the above results serve to show the completeness of the reduction.

§ Sulfinic acid obtained by decomposition of the disulfoxide (*cf.* later text). This reduction was carried out in a cabinet.

M KI-HCl, does not consume any I_2 in the presence of an excess, while cysteine under like conditions consumes the theoretical amount for oxidation to cystine (4).

In all the following determinations of intermediate oxygen, the samples were allowed to stand in a cabinet for 1 to 2 hours with 1 to 2 M KI-HCl.

A previous preparation of the disulfone (R—SO_2—SO_2—R) ((5) p. 107), which contained 6 per cent cystine, and which possessed a molecular weight of 422 from I_2 oxidation, showed less than 1 per cent reduction after 4 hours in 2 M KI-HCl solution (*cf.* also (5) p. 115). The resistance of cysteic acid and the disulfone to reduction by KI-HCl indicates that this treatment is effective only with sulfur oxidation products below the sulfonic

level (R—$\overset{O}{\underset{O}{S}}$—).[1] This is in agreement with the results of Fries

and Schürmann (7) who pointed out that aromatic sulfones, sulfonic acids, sulfonic acid esters, and sulfonic acid chlorides re-

TABLE II

Change in Optical Rotation of Solution of R—$(SO)_x$—R in N HCl (1 Gm. per 100 Cc.)

Time, *days*.....	6 (min.)	1	2	3	5	7	12	15
α_{Hg} per dm., *degrees*....	−0.302	−0.428	−0.511	−0.568	−0.662	−0.715	−0.778	−0.783
Temperature, °*C*...	28.7	29.0	29.5	29.5	30.6	28.6	30.9	32.3

sist reduction (by HBr in acetic acid), although sulfinic acids and disulfoxides are easily reduced.

Dismutative Decomposition of the Disulfoxide

Evidence from Optical Rotation—Although the disulfoxide was shown to be sufficiently stable to make possible its isolation through an aqueous acid solution (1), nevertheless decomposition occurs in such solutions, as is shown by the optical data of Table II. The negative increase in optical rotation was found to be associated with the formation of cystine which, at the end of the reaction, amounted to 29 per cent by weight or 33 moles per cent of the original disulfoxide (1 mole of R—S—S—R formed from

[1] Possibly halogen compounds (and possibly also the isomeric sulfinic

acid R—$\overset{O}{\underset{O}{S}}$—H) should be excluded, since Cleve found that several aromatic

sulfonic acid chlorides were reduced by HI in acetic acid to disulfides (6).

3 moles of R—(SO)$_2$—R). The cystine was isolated on neutralizing the solution and identified (that remaining in solution was determined by the cyanide-nitroprusside test).

The rate of the decomposition was increased by decreasing the HCl concentration, since the rotation of a 0.05 M disulfoxide solution (1.36 per cent) in 0.46 M HCl became constant after 9 days at $\alpha_{Hg}^{29} = -1.172°$ per dm. ($-0.863°$ for a 1 per cent solution).[2] On the assumption that 33 moles per cent of cystine was formed, a 0.0167 M cystine solution in 0.46 M HCl was prepared and found to possess a rotation of $\alpha_{Hg}^{26} = -1.02°$ per dm.,

TABLE III

*Cystine Formation in Aqueous Cystine Disulfoxide Solutions**

Approximately 0.2 gm. of disulfoxide per 100 cc. of solution. Cystine was determined by the cyanide-nitroprusside test (1).

pH	1	2	3	4	5	6	7	8
Solution	0.1 N HCl	0.05 M buffers			0.1 M phosphate	0.1 M phthalate	0.2 M phosphate	0.4 M borate
15 min							16	21
1 hr							29	28
4 hrs	1.8	4.3	3.4	5.9	9.7†	15‡	29	29
24 "	6.4	12	11	15	22	25	29	29

* The amount of cystine formed is expressed as percentage by weight of the original disulfoxide taken.

† pH became 4.7.

‡ pH became 5.5.

indicating a slightly negative rotation to be associated with the other products of the acid decomposition.[3]

Effect of Changes in pH—The dependence of the rate of the decomposition on acidity is further illustrated in Table III, where the extent of decomposition was determined from the amount of cystine formed. The increasingly rapid formation of relatively strong acids necessitated strong buffering, especially from pH 5 onward. Ammoniacal solutions of the disulfoxide

[2] The original value of α_{Hg}^{26} per dm. was $-0.445°$, or $-0.327°$ for a 1 per cent solution.

[3] *Cf.*, however, the optical activity of the products obtained by alkaline decomposition.

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present an anomalous behavior with respect to the decomposition; when the disulfoxide was dissolved directly in 0.16 M NH_4OH, the amount of cystine formed in 30 minutes was 9 per cent and after 3 hours only 18 per cent was present, whereas the results at pH 8 indicate the reaction to be complete in less than an hour.[4] Furthermore, when a solution of the disulfoxide in 0.1 M HCl was made 2 M in NH_3, the cystine content was only 2.0 and 2.3 per cent after 10 and 30 minutes respectively.

Decomposition in Alkaline Solution—The behavior of cystine disulfoxide in alkaline solution resembles that of aromatic disulfoxides (8) in that decomposition occurs according to the reaction

$$3R—(SO)_2—R + 4NaOH \rightarrow R—S—S—R + 4R—SO_2Na \qquad (2)$$

TABLE IV

Decomposition of Disulfoxide in Alkaline Solution

The results are calculated on a basis of 3 moles of R—(SO)$_2$—R.

Solution	Cystine formed	Acid formed	Sulfinic acid (from KI-HCl reduction)
	moles	*moles*	*moles*
0.492 gm. R—(SO)$_2$—R per 5 cc. N KOH......	0.954	3.56	3.85
0.136 " R—(SO)$_2$—R " 4 " " NaOH.....	0.846	3.98	4.00
1.361 " R—(SO)$_2$—R " 10 " 1.06 M NH$_3$..	0.922		3.93
Theory, Equation 2............................	1.0	4.00	4.00

Table IV shows the data obtained from alkaline disulfoxide solutions which were allowed to stand for about an hour. The excess alkali was then titrated with acid to the turning point of methyl red; the decrease in alkalinity was attributed to the neutralizing action of the sulfinic acid which had formed. The cystine that precipitated on standing was filtered off and that remaining in solution was determined by the cyanide-nitroprusside method; the sum of these two quantities gave the total amount of cystine formed. This was identified as *l*-cystine by quantitative application of the cyanide-nitroprusside test, iodine oxidation (molecular weight found, 238.0) (1), and optical rotation (98 per

[4] For this reason ammonia was used for neutralization of the aqueous HCl extract in the isolation of the disulfoxide (1).

cent of the theoretical). The "intermediate oxygen" in the filtrate was determined by the KI-HCl reduction and calculated as sulfinic acid.

The decomposition was also carried out in an ammoniacal solution by dissolving the disulfoxide in an excess of ammonia, evaporating to incipient dryness at about 30°, adding water, and repeating until no odor of ammonia remained; the cystine was then filtered off (this procedure yielded a solution 0.25 M in sulfinic acid and about 0.001 M in cystine).

Isolation of Sulfinic Acid—Although sulfinate solutions containing relatively small amounts of cystine were obtained in the above manner, nevertheless, the attempts to isolate the pure sulfinic acid were not uniformly successful. The method utilized by Schubert (2) of precipitating a barium salt by alcohol yielded a material containing 44.2 per cent Ba and consuming 1 gm. atom of O (I_2) per 278 gm.; theoretical for R—$SO_2Ba \cdot H_2O$ is 44.8 per cent Ba; molecular weight, 306 gm. Removal of barium by an equivalent amount of H_2SO_4 and evaporation of the filtrate *in vacuo* yielded a glassy residue which, on rubbing with alcohol or acetone, formed an extremely hygroscopic powder. This material possessed analytical values comparable with a sulfinic acid containing 7 per cent cystine (*i.e.*, S content, I_2 oxidation, reduction by KI-HCl, and cyanide-nitroprusside test for cystine).

Treatment of a sulfinate solution with copper acetate formed a deep blue solution. After removing a small amount of gray precipitate, an apple-green flocculent precipitate was thrown out by the addition of alcohol containing a few drops of acetic acid. After filtering and drying, the material was suspended in water, and the copper removed by H_2S;[5] found, 24.8 per cent Cu. When the filtrate was treated as before, a product was isolated which contained 4 per cent cystine according to the nitroprusside test and which possessed a molecular weight of 157 by the I_2 oxidation, corresponding to 96.5 per cent R—$SO_2H \cdot H_2O$ and 3.5 per cent cystine.

The most successful isolation resulted from the filtrate of an ammoniacal decomposition of 2.72 gm. of disulfoxide. The slightly yellow filtrate from the cystine was decolorized with

[5] Treatment of a sulfinate solution with H_2S for 25 minutes occasioned only a 1.8 per cent loss in intermediate oxygen.

charcoal, the theoretical amount of HCl added, and the solution then evaporated to dryness. After dissolving in about 4 cc. of water, about 5 cc. of alcohol were added, which caused separation into two layers. Water was then added to the hot liquid (about 60°) until a homogeneous solution was again obtained. On cooling, tiny octahedral crystals separated out, amounting to 0.91 gm., or 48 per cent of the theory. This material was not especially hygroscopic and lost no weight on drying at 100° *in vacuo* over P_2O_5.

The following evidence indicated that this substance was the sulfinic acid, $C_3H_7O_4NS$; molecular weight, 153.1.

> Theoretical.   S 20.94, N 9.15
> Found.   " 21.30, " 9.15[•] (Folin's micro-Kjeldahl)

Acid value (methyl red as indicator), 1 equivalent per 154.9 gm.; KI-HCl reduction, 1.5 gm. atoms of O liberated per 152.0 gm. (*cf.* Table I); I_2 oxidation, 1 gm. atom of O consumed per 151.4 gm. The cyanide-nitroprusside test was negative; *i.e.*, less than 0.2 per cent of cystine present.

This material decomposed sharply with much foaming at 152–153° (corrected); Schubert's sulfinic acid at 152–153°; sulfinic acid from copper salt at 147–148°. (Preparation of the sulfinic acid ($R—SO_2H·H_2O$), according to Schubert (2), yielded in our hands a product which possessed the following molecular weights: 165 by I_2 oxidation, 153 by KI-HCl reduction, 160.6 by titration with alkali.)

The sulfinic acid possesses a pK of approximately 2.1 (determined colorimetrically on a half neutralized 0.065 M solution and with 1.5 as the pK of thymol blue).

The optical rotation of sulfinic acid solutions depends on the acidity, as is shown by the values of $[\alpha]_{Hg}^{27-28}$, obtained on the following solutions (1 gm. of $R—SO_2H$ per 100 cc.): +33.4° in 1 M HCl; +11° in water; −5.4° in a half neutralized solution; −23.8° in a solution of the sodium salt. The sulfinic acid prepared according to Schubert possessed somewhat different values; $[\alpha]_{Hg}^{25}$ was +10.9° for a solution containing 0.827 gm. per 100

[•] The author wishes to thank Dr. Grace Medes of this Institute for performing the N determination.

cc. of M HCl, and $-12.7°$ for 1.103 gm. dissolved in 100 cc. of water.

Acid Decomposition—The decomposition of the disulfoxide in acid solution is somewhat obscure because of a decrease in available intermediate oxygen, although the same amount, 33 moles per cent, of cystine was formed. Fig. 1 shows the course of the decomposition in 0.1 N HCl.[7] Data obtained from aqueous and acidified solutions of the disulfoxide are summarized in Table V.

In Solutions 1, 2, and 3 aqueous suspensions of the disulfoxide were boiled for about an hour (some cystine crystals precipitated); neutralization of the now acid solution to the turning point of methyl red yielded the amount of acid formed. The cystine was
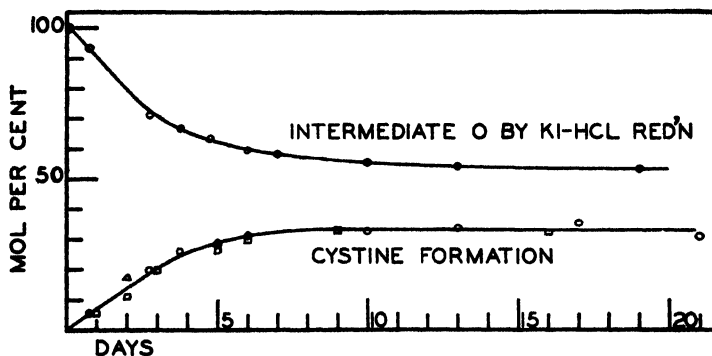
Fig. 1. Decomposition of cystine disulfoxide in 0.1 N HCl (0.332 gm. per 100 cc.).

filtered off and the amounts of cystine and intermediate oxygen in the filtrate determined as before. Solution 4 was allowed to stand for 13 days at room temperature (the reaction was completed in 9 days according to the optical rotation). Solution 5 was a suspension of the disulfoxide kept at about 50°, in which the sulfinic acid was neutralized, as formed. In determining the I₂ necessary for oxidation of the acid intermediates in the filtrates to cysteic acid (1) (deducting that consumed by the cystine present), it was found that while the oxygen consumed after 24 hours corresponded to the acid formed, there was, however, a

[7] Although a monomolecular reaction is indicated by the results in Fig. 1, further data are necessary before this can be asserted positively.

slow additional iodine consumption over a period of several days, despite the fact that 24 hours are sufficient for oxidation of cystine, the disulfoxide, or the sulfinic acid to cysteic acid. This behavior suggests the presence of an intermediate which is more resistant to oxidation by iodine than either the disulfoxide or the

TABLE V

Decomposition of Cystine Disulfoxide in Aqueous and Acid Solutions
The results are calculated on the basis of 3 moles of disulfoxide.

Solution No.		Acid formed	Cystine formed	Interme-diate oxygen	O consump-tion (from oxidation by I_2)
		equivalents	*moles*	*gm. atoms*	*gm. atoms*
1	Aqueous suspension	2.86	0.894	3.468	
2	" "	3.03	0.796*	3.270	4.34
3	" "	3.04	1.044	3.288	
4	0.46 N HCl	3.48	1.116	3.222	4.21†
5	Progressively neutralized	3.11	1.050	3.510	3.91‡
	Theory, Equation 2	4.00	1.00	6.00	4.00
	" " 3	3.00	1.00	3.50	4.00

* Some cystine was accidentally lost.
† 3.13 gm. atoms of O consumed after 1 day in oxidation by excess I_2.
‡ 3.08 gm. atoms of O consumed after 1 day in oxidation by excess I_2.

$$\text{sulfinic acid } (R\text{—}\overset{O}{\underset{O}{S}}\text{—OH}) \text{ and which might well be an isomeric}$$

$$\text{sulfinic acid, } R\text{—}\overset{O}{\underset{O}{S}}\text{—H. \quad Equation 3}$$

$$3R\text{—}(SO)_2\text{—}R + 2H_2O \cdot \cdot R\text{—}S\text{—}S\text{—}R + 2R\text{—}SO_2H + R\text{—}S(O)H$$
$$+ R\text{—}SO_3H \tag{3}$$
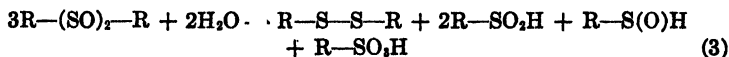
agrees reasonably well with the experimental data of Table V, if it is assumed that the isomeric sulfenic acid, R—S(O)—H, does not enter into the acid titration, and that it forms on oxidation the isomeric sulfinic acid which would thus account for the slowness of the last part of the oxidation. Further differences between the acid intermediates arising respectively from acid and alkaline decomposition were apparent from the negative rotation of the former as contrasted with the positive rotation of the latter

in HCl solution and also from the fact that on standing 8 months
in 0.5 м HCl the former lost 83 per cent of its intermediate oxygen,
while the latter (sulfinic acid) lost only 12 per cent. There was
no appreciable increase in the cystine content in either case.

Reaction of Disulfoxide with Thiol Compounds

Reaction with Cysteine—The disulfoxide reacts with cysteine
in either acid or alkaline solution in accordance with the reaction

$$R—(SO)_2—R + R—SH \rightarrow R—S—S—R + R—SO_2H \qquad (4)$$
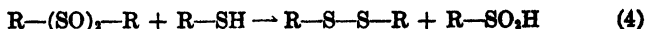
as is shown by Table VI. This behavior is similar to that ex-
hibited by aromatic disulfoxides (9). The polariscopic data,

TABLE VI

Reaction of Equimolar Amounts of Cysteine and Cystine Disulfoxide

Solution	Cystine formed	Acid formed	Sulfinic acid (from KI-HCl reduction)
	moles	*equivalents*	*moles*
0.2 м HCl	1.01	1.12	0.96
Aqueous*	0.87	0.92	0.96
1 м NaOH	1.00	0.90	0.98

* An aqueous suspension of the disulfoxide was heated with a solution
of cysteine hydrochloride.

illustrated in Fig. 2, showed the reaction to be very rapid and
apparently finished in 6 to 10 minutes, at the time when the first
readings were taken. The conclusion, that the acid formed is
the sulfinic acid, was supported by the analytical evidence and
by the fact that the rotation was less than that of a corresponding
cystine solution, thus indicating the presence of a substance with
a positive rotation. The sulfinic acid which was formed reacted
slowly with cysteine (*cf.* Solutions 2 and 3 in Fig. 2). The analyt-
ical evidence from these solutions, after the rotation had become
constant (determinations of cystine, acid titration, and KI-
HCl reduction), agreed with the assumption that the sulfinic
acid reacted with excess cysteine to form cystine (R—SO$_2$H +
3R—SH→2R—S—S—R + 2H$_2$O). In Solution 3 (after 450
hours) no acid products remained beyond the added HCl; the
total cystine formed was 97.5 per cent of the theoretical; no

intermediate oxygen could be detected and the solution was 0.0434 M in cysteine.   The reaction of the sulfinic acid with cysteine appears to be even slower in less acid solution; a neutralized 0.0262 M solution of the isolated sulfinic acid previously described was made 0.0785 M in cysteine and found to possess an initial rotation of $-0.18°$ per dm., which after 21 days had become only $-0.29°$ per dm. (the pH was approximately 4.5).
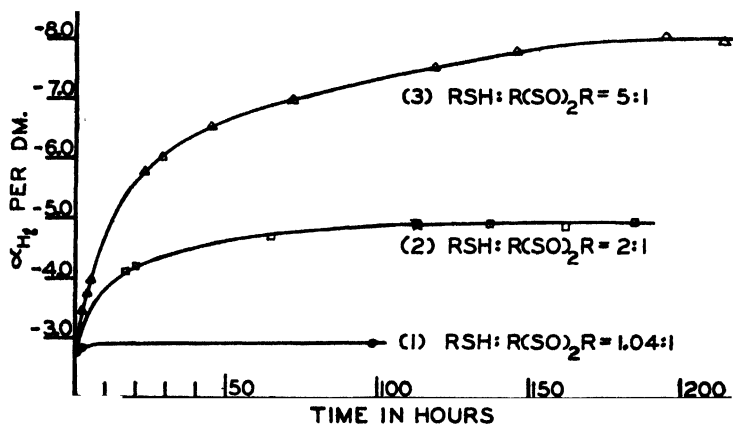
Fig. 2. Reaction of cystine disulfoxide with cysteine.   Solutions 1 and 2 were 0.025 M disulfoxide solutions in 0.22 M HCl; Solution 3 was a 0.05 M disulfoxide solution in 0.46 M HCl.   The rotations of Solutions 1 and 2 were doubled for comparison with Solution 3.   The original value was $-2.72° \pm 0.02°$ per dm. for each of the three solutions.   The rotations (α_{Hg}^{20} per dm.) of corresponding cystine solutions were as follows: $-2.99°$ for 0.05 M R—S—S—R in 0.45 M HCl and $-1.57°$ ($\times 2 = -3.14°$) for 0.025 M R—S—S—R in 0.22 M HCl.

Reaction with H_2S, Thioglycolic Acid, and p-Thiocresol—The reaction of the disulfoxide with cysteine (Equation 4) is apparently the prototype of reactions with other thiol compounds.   Thus it was found that H_2S quickly effected solution of an aqueous suspension of the disulfoxide, and that equimolar amounts of the disulfoxide and thioglycolic acid reacted practically instantaneously according to the optical rotation; α_{Hg}^{20} was $-1.08°$ per dm. for an 0.05 M solution of each component in 0.42 M HCl.   For neutralization of each of the above solutions the theoretical amount of alkali required by reactions similar to Equation 4 was

used; there was no precipitation after neutralization. However, further work is necessary for identification of the reaction products (*i.e.*, R—S—SH and R—S—S—R′). The formation of the sulfinic acid was also indicated by the unchanged intermediate oxygen content of the solutions. Titration of the excess alkali after the reaction of the disulfoxide with thioglycolic acid in alkaline solution was accompanied by a strong H_2S odor, although in other respects the reaction was similar to that in acid solution.

The addition of 0.56 mM of the disulfoxide in small portions to an alkaline solution of 0.56 mM of *p*-thiocresol resulted in a milky suspension and crystal separation. Upon neutralization, 0.54 mM of acid was found to have formed. The filtrate from the precipitate contained 0.58 mM of R—SO_2H according to the KI-HCl reduction and about 0.06 mM of thiol compound. The precipitate was resolved by absolute alcohol into two fractions consisting of 0.242 mM cystine and 0.246 mM *p,p′*-ditolyldisulfide; this latter compound was identified by its melting point. Found, 46°; Beilstein, 46° and 48°. The reaction thus appears to be

$$2R—(SO)_2—R + 2Ar—SH \rightarrow R—S—S—R + Ar—S—S—Ar + 2R—SO_2H \quad (5)$$
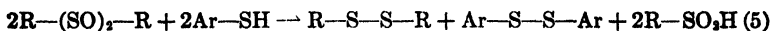
(Ar = aryl). The mechanism by which the two disulfides arise offers interesting possibilities in view of the apparently simple interaction of the disulfoxide with the other thiol compounds studied.

Reaction with NaCN—The disulfoxide was found to react with sodium cyanide in the same manner as cystine.

$$R—(SO)_2—R + NaCN \rightarrow R—SCN + R—SO_2Na \quad (6)$$
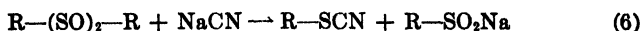
The addition of an equimolar amount of NaCN to an aqueous suspension of the disulfoxide resulted in a clear solution from which the R—SCN was isolated (about 50 per cent of the theoretical) by Mauthner's technique (10). This was identified by its decomposition point (220°) and other properties. An impure sulfinic acid contaminated with cystine (which doubtless resulted from the H_2S treatment used for removal of Cu) was also obtained. This reaction of the disulfoxide with cyanide is of importance in the cyanide-nitroprusside test used to determine the presence of cystine, since it eliminates the dismutative decomposition of the disulfoxide during the test.

Reaction with Phospho-18-Tungstic Acid and Reaction Mech-

anism of Dismutative Decomposition—Fresh solutions of the disulfoxide slowly develop a blue color with phosphotungstate (Folin and Marenzi uric acid reagent) at pH 5.2 under the conditions established by Shinohara (11). At room temperature the color development is rather slow. However, on heating a 0.002 M disulfoxide solution with the phosphotungstate reagent on a boiling water bath, the color development reached a maximum within an hour, corresponding to an 0.00081 M cysteine solution[8] treated under the same conditions. From the stoichiometrical relationships the following reactions seem plausible,

$$2R—(SO)_2—R + H_2O \rightarrow 2R—SOH + 2R—SO_2H \quad (7)$$
$$2R—SOH \rightarrow R—SH + R—SO_2H \quad (8)$$
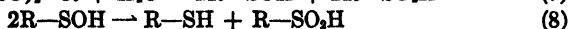
and it appears that the cysteine is responsible for the color development rather than either the disulfoxide or the sulfenic acid. (The sulfinic acid does not respond to the test.) Furthermore, the addition of bisulfite, at the end of the reaction, qualitatively increased the color, indicating formation of cystine by the action of the phosphotungstate reagent (13). If the phosphotungstate were not present, the cysteine would have reacted with the disulfoxide according to Equation 4, which when combined with Equations 7 and 8 would yield Equation 2 (the decomposition shown to occur in alkaline solution).

Structure—The structure assigned to this new compound has been, provisionally, that of the true disulfoxide, R—SO—SO—R, despite the general acceptance of a thiol sulfonate (R—S—SO_2—R) structure for aromatic disulfoxides. The ease of reduction of this compound with aqueous KI-HCl solution at room temperature compared with the stability of a fully saturated sulfur atom (as the thiol sulfonate formula would imply) strongly suggests the disulfoxide structure. On the other hand, the reactions with thiol compounds and NaCN apparently favor the thiol sulfonate structure. In these latter cases, however, as in any aqueous

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[8] The author wishes to thank Dr. Grace Medes for performing the colorimetric determinations. Dr. Medes also found that no color was developed when bisulfite was added to a fresh disulfoxide solution. The action of bisulfite in this case is probably similar to that of sodium cyanide, and to the action of these reagents on cystine (12); *i.e.*, R—(SO)_2—R+ NaHSO_3 \rightarrow R—S—SO_3H + R—SO_2Na.

reaction, the lability of these intermediate compounds must be considered in view of the possibilities of hydrolysis as well as of oxidation and reduction. It seems very possible that there may be an equilibrium between the two forms of the disulfoxide.

The author wishes to express here his thanks to Dr. G. Toennies of this Institute and to Professor E. C. Wagner of the University of Pennsylvania for their interest and suggestions in the course of this work.

SUMMARY

The various chemical properties of the disulfoxide of cystine have been investigated, including: (1) the development of a method of determining the intermediate oxygen of cystine intermediates, (2) the dismutative decomposition of aqueous solutions, (3) the isolation of a sulfinic acid as one of the products from alkaline decomposition, (4) the reaction of the disulfoxide with various thiol compounds, sodium cyanide, and phospho-18-tungstic acid.

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THE EFFECT OF TISSUE EXTRACTS ON ESTERIFICATION OF CHOLESTEROL IN BLOOD SERUM*

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In a recent publication (1) attention was called to an apparent discrepancy between the finding in this laboratory of a marked esterification of free cholesterol on incubation of human blood serum and results reported by Shope (2), who concluded that cholesterol esters were hydrolyzed when cow serum was incubated with cholesterol-free, saline extracts of various animal tissues.[1] As the proportion of free and combined cholesterol of the serum was not altered in Shope's procedure, the reaction should have proceeded in the same direction both in his experiments and in those carried out here. The lack of agreement cannot be due to dilution of serum with tissue extracts, since, just as in undiluted serum, free cholesterol was esterified on incubation of serum which had been diluted with several times as much saline as was employed by Shope in the form of tissue extract.

As the point involved is of fundamental importance in regard to the cholesterol esterase of blood serum, experiments similar to those of Shope were carried out in an attempt to explain the

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* This investigation was made possible by the support of the Josiah Macy, Jr., Foundation.

[1] Shope was stimulated to carry out these experiments by his observation that cholesterol esters were usually much reduced or absent in blood serum taken post mortem from animals. On the basis of this finding he postulated the presence of a very active cholesterol ester-splitting substance in animal tissues. In the course of an investigation being carried out in this laboratory the concentration of total and free cholesterol has been determined in a considerable number of samples of serum taken post mortem from healthy, human subjects who had died suddenly. In over 80 per cent the ratio of combined to free cholesterol was within normal limits.

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discrepancy. It has proved impossible to confirm Shope's conclusion; no splitting of cholesterol esters occurred when blood serum was incubated with tissue extracts. On the contrary, in most instances a definite esterification of free cholesterol took place.

<div align="center">EXPERIMENTAL</div>

The normal animals, used in the preparation of tissue extracts, were killed by decapitation, the blood being drained as completely as possible. Samples of various tissues were removed at once, weighed, and ground with sand, 5 cc. of physiological salt solution per gm. of tissue being added during the grinding. The suspensions were centrifuged (except in Experiment 8) and then filtered. 2 volumes of filtrate were mixed thoroughly with 3 volumes of serum and samples of the mixture were extracted for cholesterol determination. Other portions were incubated at 37° in tightly stoppered test-tubes for 3 and 24 hour periods, after which the concentration of total and free cholesterol was determined as before. In control samples physiological saline was substituted for tissue extracts. The foregoing procedure for preparation of extracts is identical with that employed by Shope except for the use of sand in grinding the tissues, and 'he centrifuging, which greatly facilitated the filtration, especially in the case of spleen. As a result of a discussion with Dr. Shope (see below) it has become evident that the writer's experiments differed from his also in regard to the control analyses before incubation, which were omitted in Shope's work. The same tissues employed by Shope, with the exception of testicle, were studied. Six experiments with beef serum and guinea pig tissue (the combination used in most of Shope's work), and one experiment each with sheep serum and guinea pig tissue, human serum and mouse tissue, and human serum and rat tissue were carried out. The beef and sheep bloods were obtained at the slaughter-house, brought to the laboratory, and centrifuged immediately. They were, in most instances, still warm when they arrived.

As in Shope's work, no preservative was employed except in one of the beef serum experiments where 1 per cent of a 10 per cent alcoholic solution of thymol was added to each of the serum-tissue extract mixtures. In this experiment no change in the

proportion of total and free cholesterol occurred during incubation either in the control or tissue extract samples, and it was found subsequently that thymol inhibits the esterification of free cholesterol almost completely in beef serum.[2]

The method of Schoenheimer and Sperry (3) was used for all cholesterol determinations, but in the experiments with beef and sheep serum it was necessary to extract 0.4 cc. of the serum-tissue extract mixtures instead of the usual 0.2 cc. The concentration of free cholesterol varied from 19.0 to 24.5 mg. per 100 cc. in the six samples of beef serum studied, while the single sample of sheep serum contained only 12.7 mg. per 100 cc. These low concentrations were reduced by two-fifths in diluting with tissue extract and still further reduced by the esterification which occurred during incubation in most cases. Hence, even with the double amount of mixture taken for analysis, most of the free cholesterol readings fell between 60 and 75 per cent light transmission, a range in which the error of reading the Zeiss Pulfrich photometer is relatively high. In order to increase the accuracy as much as possible duplicate determinations were made in all the experiments with beef and sheep serum (except in Experiments 1, 2, and 7, where not enough spleen extract was available). The error, expressed as percentage variation, was relatively high, as was to be expected. In all, 156 determinations of free cholesterol were made in duplicate. Of these 56 per cent varied from their means by less than 2 per cent, 76 per cent by less than 3 per cent, and 91 per cent by less than 5 per cent. However, the actual variation in absolute amount was relatively small; 84 per cent of the values varied from their means by 0.5 mg. per 100 cc. or less, and 97 per cent by 1.0 mg. per 100 cc. or less. Better agreement cannot be expected in determining such small amounts.

DISCUSSION

Altogether 835 individual determinations of cholesterol were made in the course of this investigation. The results, which are essential to the problem in hand, are summarized in Table I in terms of percentage esterification (percentage decrease in con-

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[2] Thymol inhibited the esterification to some extent in human serum, though marked esterification still occurred, as was found early in the study of cholesterol esterase (1).

centration of free cholesterol from the original level during incubation).

The data obtained on 24 hour incubation will be discussed first. Without exception in all of the 56 instances in which tissue extract was incubated with blood serum, an esterification took place. In a few cases the change was less than the experimental error of the free cholesterol determination, especially in Experiments 2

TABLE I

Percentage Esterification of Free Cholesterol* in Blood Serum-Tissue Extract Mixtures

Experiment No.		1	2	3	5†	6	7	8	9
Serum..		Beef					Sheep	Human	
Tissue		Guinea pig						Mouse	Rat
3 hr. incubation	Control	3.8	7.0	1.4	8.0	−3.8	23.3	20.6	0.7
	Liver	−0.6	8.8	2.5	5.3	0.7	1.1	13.1	8.2
	Kidney	6.3	0.6	−0.6	0.6	3.6	−1.6	11.8	14.5
	Muscle	2.1	4.3	2.1	7.5	−2.1	8.8	12.9	1.8
	Lung	−1.0	0.5	9.5	5.6	7.3	10.2	15.3	15.9
	Brain	0.6	0.6	0.6	4.6	2.7	4.4	3.0	9.5
	Spleen	2.8	3.7	5.4	4.2	1.2	5.6	13.2	2.7
	Heart	6.4	1.9	4.2	4.1	2.0	10.1	18.7	18.3
24 hr. incubation	Control	20.5	10.9	24.6	28.0	6.9	27.9	25.0	40.2
	Liver	30.8	8.8	17.4	18.2	6.2	16.8	22.8	31.2
	Kidney	39.2	4.9	12.0	9.0	15.8	27.0	7.6	31.4
	Muscle	17.1	7.8	20.4	16.3	11.4	31.9	17.3	40.3
	Lung	46.9	10.2	17.5	17.7	26.4	37.2	24.3	36.5
	Brain	17.2	1.7	15.8	10.7	10.9	18.5	3.4	29.7
	Spleen	14.2	0.6	19.7	12.7	6.6	7.4	15.2	19.1
	Heart	29.9	12.1	18.0	17.2	12.8	27.3	7.5	9.7

* Percentage decrease in free cholesterol during incubation.
† In Experiment 4 thymol was added. No change occurred; see text.

and 6 where the degree of esterification was small in the control samples; but the results as a whole show conclusively that, qualitatively, esterification of serum-free cholesterol occurs in the presence of tissue extracts, just as it does when blood serum is incubated alone.

From the quantitative point of view the data are more difficult to interpret. With lung extracts almost as much or more esterifi-

cation occurred in six out of eight experiments than in the control samples. In thirty-four of the remaining forty-eight instances the degree of esterification was less in the presence of tissue extract than in the control samples, in nine it was higher, while in five there was little difference. All but two of the fourteen cases, where tissue extract did not exercise an apparent inhibitory effect, occurred in Experiments 1, 6, and 7. In Experiment 7 the concentrations of free cholesterol were exceptionally low (minimum 6.2 mg. per 100 cc.) and the probable error was consequently large. In Experiment 6 agreement between duplicates was especially poor in the determination of free cholesterol in the control sample after 24 hours incubation and the percentage esterification value for this sample may be considerably in error. (Calculated from the lower free cholesterol analysis the percentage esterification would be 14.6 per cent instead of 6.9 per cent.) Since most of the exceptions may well have been due to experimental error, it appears justifiable to conclude that tissue (except lung) extracts at least tended to inhibit esterification. The inhibitory tendency may possibly have been associated with the formation of sediment, which occurred to a greater or less extent in most cases during incubation, and which was probably due in large part to the separation of emulsified material from the tissue extracts. (Some of the sediment may have represented the growth of microorganisms, though in the experiment in which thymol was added considerable flocculation also occurred.) It was observed early in the study of cholesterol esterase (1) that addition of cholesterol emulsions to serum inhibited esterification and it was thought that the enzyme was carried down with the sediment which formed in all cases during incubation. However, in the present investigation no definite correlation could be made out between the amount of sediment which appeared to be present and the degree of esterification.

In most cases in experiments with beef and sheep serum the changes after 3 hours incubation were less than the experimental error. However, in only five instances out of 56 in which serum was incubated with tissue extract was there an apparent small negative esterification (hydrolysis of cholesterol esters), and in one of these (muscle, Experiment 6) the control sample showed

an even larger apparent splitting.[3] It is highly unlikely that such a large proportion of the samples showed an apparent esterification entirely by chance, but, whether or not it be admitted that some esterification occurred, it is quite certain that there was no hydrolysis of cholesterol esters such as was claimed by Shope to occur during incubation for 3 hours.

The opinion that an actual discrepancy existed between the results reported by Shope and those from this laboratory was based on Shope's statement that his tissue extracts were cholesterol-free. The extracts used in this investigation, though prepared by essentially the same technique, contained small but definite amounts of cholesterol, with the probable exception of those from muscle. The evidence for this conclusion is twofold: In the first place in all but six out of 63[4] instances the serum-tissue extract mixture contained more total cholesterol than its control. Four of the six exceptions were in samples containing muscle extract. Secondly, in all cases save one (muscle extract) the ratio of combined to free cholesterol was lower in the samples containing tissue extracts than in their controls. This finding, indicating an increased proportion of free cholesterol, would be expected if some tissue cholesterol, which is largely in the free state, were carried into the mixtures by the extracts. An increased proportion of free cholesterol might be expected to promote esterification, and it would seem possible, if Shope's extracts contained no cholesterol, that the discrepancy might be explained on this basis. Such an explanation is unlikely, however, since the amount of free cholesterol in the extracts was small at the most in proportion to that present in the serum. Muscle extracts, in the presence of which a relatively high degree of esterification occurred in most instances, contained little if any cholesterol.

The foregoing results and discussion were submitted to Dr. Shope for his comment, in the hope that a probable explanation for the discrepancy might be found. Such has been the case.[5]

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[3] This is the only case in which an apparent splitting of cholesterol esters has been observed during incubation of serum alone or serum diluted with saline or distilled water. The change is within the experimental error of the procedure.

[4] The thymol experiment is included in this analysis of the data.

[5] The author is indebted to Dr. Shope for his frank, courteous, and friendly discussion, which has led to the probable explanation of an apparent discrepancy.

Dr. Shope pointed out that the writer had misinterpreted his experiments in making the statement ((1) p. 475) that, "Serum was not incubated alone [by Shope]." The author took it for granted erroneously, in making this statement, that Shope's control analyses had been made on unincubated samples of serum diluted with saline, since, in view of the author's findings in regard to esterification of cholesterol in serum, such analyses are essential to the conclusion that hydrolysis of cholesterol esters occurred. Actually no analyses were made before incubation. The control samples were incubated just as the mixtures of serum and tissue extracts and analyzed only *after* incubation, it being assumed, in the absence at that time of evidence to the contrary, that no significant change in the proportion of combined and free cholesterol would occur on incubation of serum diluted with saline. It is entirely possible and indeed probable, therefore, that an esterification occurred in Shope's control samples just as in the present study, and that the smaller proportion of combined cholesterol, found in tissue extract mixtures after incubation, represented the inhibitory tendency which was noted in the author's investigation.

Dr. Shope also called attention to another difference in the two series of experiments. In his work the serum was obtained from lactating cows on a full diet, while in the present study the beef and sheep serum was obtained at the slaughter-house from animals in an unknown state of nutrition. Since the esterification of cholesterol on incubation of serum has been observed in all samples of serum (human, beef, and sheep) so far investigated, it appears to be a general phenomenon and it hardly seems likely that serum from lactating cows would be an exception.

Three incidental observations were made in the course of this investigation: Beef serum appears to contain a somewhat smaller proportion of free cholesterol than does human serum. In the six samples analyzed the ratio of combined to free cholesterol varied from 3.35 to 3.89. Such high ratios are almost never encountered in human blood serum. Second, the percentage esterification was almost as great in the control samples (serum diluted with saline) as in undiluted serum, which was also incubated in all of these experiments. The average esterification in 24 hours for the control samples was 23.0 per cent, and for undiluted serum, 25.2 per cent. Third, the percentage esterifica-

tion appears to be less in beef serum than in human serum, though, in view of the wide variation among individual sera, a definite conclusion to this effect cannot be drawn on the basis of the five samples studied. The average esterification for the undiluted beef sera was 20.6 per cent as compared to an average of 57 per cent in the case of human sera (1). The difference is probably not due to the longer period of incubation (3 days) in the case of the human sera, since other evidence indicates that the esterification is practically complete at the end of the 1st day.

SUMMARY

Saline extracts of various tissues from different species of animals were incubated with beef, sheep, and human blood serum. An esterification of free cholesterol occurred, just as when serum was incubated alone, but in most instances the tissue extracts appeared to inhibit the reaction to some extent.

The results are not in agreement with Shope's conclusion (2) that cholesterol esters are hydrolyzed under essentially the same conditions. A probable explanation of the apparent discrepancy is presented.

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THE ISOLATION OF A FOURTH CRYSTALLIZABLE JACK BEAN GLOBULIN THROUGH THE DIGESTION OF CANAVALIN WITH TRYPSIN

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Through work in this laboratory it has been shown that the jack bean contains three crystallizable globulins and a fourth globulin which does not crystallize. These are respectively, concanavalin A, recently identified by us as a hemagglutinin (1–3); concanavalin B (1, 2); urease (4); and canavalin (1, 2). Last summer we discovered that a sample of canavalin which had been contaminated by bacteria deposited crystals of a globulin hitherto unknown to us. We have failed repeatedly to obtain this crystalline material from unaltered canavalin, but recently have prepared it through the action of trypsin on canavalin. If one adds 1 volume of 1 per cent Fairchild's trypsin to 1 volume of 6 per cent canavalin at pH 6.5 and maintains the solution at 37°, crystals of the new globulin will begin to separate out after 1 or 2 hours. After incubation overnight the process will be finished. The yield of crystals is increased by cooling the warm solution.

The crystals, which may have a diameter of more than 1 mm., are rhombohedral and belong to the hexagonal system. They are uniaxial and optically negative with weak double refraction. There is a very low birefringence. The obtuse angle on the rhombohedral face is 110°. A photograph is shown in Fig. 1. The material gives a strong test for unoxidized sulfur and for tyrosine, but gives no Hopkins and Cole test, whereas the original canavalin contains 0.2 per cent tryptophane. Carbohydrate is absent. The isoelectric point of the crystals in 0.1 N acetate buffer is at pH 4.8, as determined by cataphoresis. The globulin is denatured incompletely by boiling, or by contact with 0.1 N

sodium hydroxide. It is resistant to 0.1 N hydrochloric acid. It is hardly affected by incubation overnight at 37° with either pepsin or trypsin.

The most interesting property of the new globulin is its solubility in distilled water at pH 6.5 and its insolubility in 0.2 to 1.0 per cent sodium chloride. If one dialyzes the globulin until
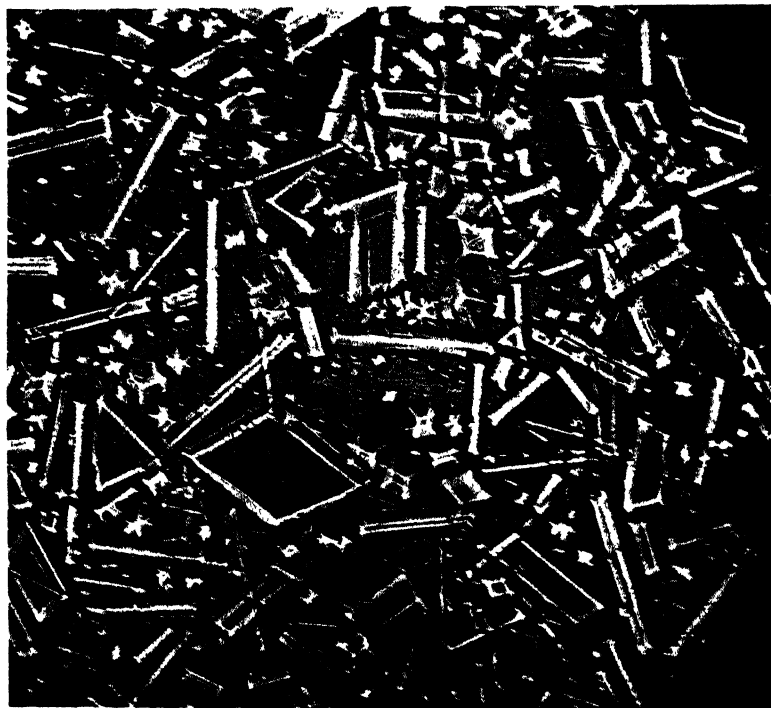
Fig. 1. Crystals of new jack bean globulin

it is salt-free, the crystals slowly dissolve. The solution thus formed will give a crystalline precipitate if one adds a small amount of sodium chloride or calcium chloride solution. Upon adding an excess of 5 per cent salt solution the crystals dissolve. The globulin behaves in a similar manner on the acid side of the isoelectric point, but not at the isoelectric point itself.

It is not yet possible to say whether the new globulin exists as such in the canavalin fraction or whether it is formed *de novo*

by the proteolysis of canavalin. It would appear reasonable that the action of trypsin is to digest away some protein which prevents the new globulin from crystallizing. This assumption is supported by the observation that if a solution of this globulin is mixed with excess of canavalin, the crystals cannot then be obtained by dialysis. The curve in Fig. 2 represents the digestion of canavalin by trypsin at pH 6.3 and 37° as followed by alcohol titration (5). It indicates that a considerable amount of hydrolytic action must precede the separation of the crystals.
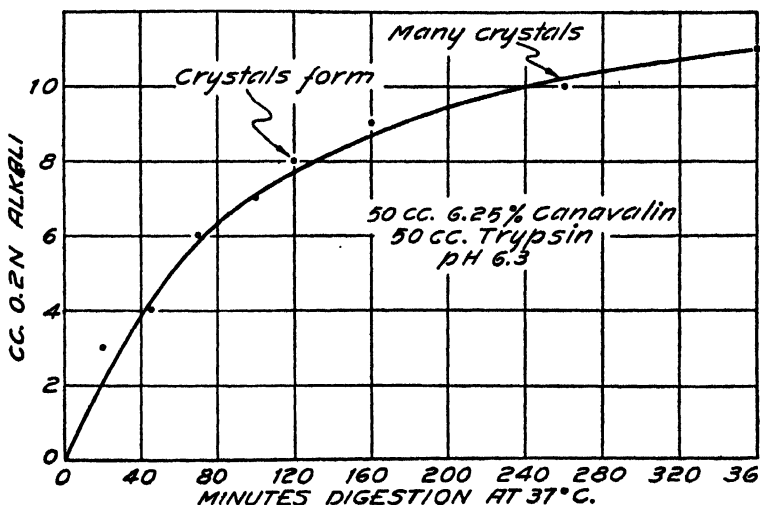
FIG. 2. Digestion of canavalin by trypsin

Ficin, papain-cysteine, and bromelin-cysteine acting upon canavalin probably give rise to the same product as trypsin. Ficin added to canavalin at pH 5.9 rapidly produces a turbidity in a manner similar to that of trypsin acting upon casein. Pepsin produces a crystallizable substance which may be the new globulin in impure condition. It has been found that the yield of crystals obtained by allowing 5 mg. of ficin[1] to act upon 1462 mg. of canavalin for 48 hours at 37° amounts to more than 80 per cent.

For the convenience of those who wish to employ canavalin for the preparation of the new globulin, we describe below a method for canavalin.

[1] This enzyme preparation was kindly supplied by Professor B. H. Robbins of Vanderbilt University.

To 100 gm. of jack bean meal add 500 cc. of 32 per cent acetone and stir. Filter overnight in an ice chest. The filtrate can be used for isolating urease crystals. The residue on the filter is mixed with 500 cc. of 30 per cent alcohol and filtered at room temperature. The filtrate is discarded. The residue on the filter is stirred up with 400 cc. of 1 per cent sodium chloride containing 0.1 per cent neutral phosphate. The material is filtered and the residue is extracted with 250 cc. of 5 per cent sodium chloride and filtered. The combined filtrates are dialyzed in three collodion bags for 2 days against several. changes of distilled water, with toluene as preservative. At the end of this time the crystalline precipitate of concanavalin A is filtered off. To the filtrate one adds enough normal acetic acid to bring the material to about pH 5.1. The precipitate is filtered off, placed in a beaker, and enough 0.1 N alkali is added to give a neutral reaction. The dissolved canavalin is then filtered from crystals of concanavalin B. The canavalin is once more precipitated by adding acetic acid, redissolved by alkali, and filtered.

SUMMARY

The preparation of a new crystallizable jack bean globulin through the action of trypsin upon canavalin is described. The new globulin is soluble in distilled water and in 5 per cent sodium chloride, but is insoluble in 0.2 to 1.0 per cent salt at pH 6.5.

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STROPHANTHIN

XXXIII. THE OXIDATION OF ANHYDROAGLUCONE DERIVATIVES

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The more recent interpretation of the course of the oxidation with permanganate in alkaline solution of monoanhydrodihydrostrophanthidin and monoanhydrodihydrodigitoxigenin after saponification has made it quite certain that the double bond of these substances under such conditions must lie between carbon atoms (14) and (15) of the sterol skeleton[1] (I). The oxidation product in each case was shown to be a dihydroxy derivative which on further oxidation with chromic acid yielded a keto acid with ring cleavage.[2] Since reduction of the carbonyl groups of these acids to hydroxyl resulted in prompt lactonization, it was apparent that a 5-membered ring, Ring D, was the one which was opened and therefore between carbon atoms (14) and (15).

In the meantime, however, it had been found that the reaction takes a different course if permanganate in acetic acid is employed. Under these conditions, monoanhydrodihydrostrophanthidin yields an acid, $C_{23}H_{32}O_7$, by the addition of 2 atoms of oxygen. Here, as in the above case, 1 oxygen atom is accounted for by that required for oxidation of the aldehyde group on carbon atom (10) to carboxyl. The function of the second oxygen atom, at first difficult to interpret, was later explained by the study of the following series of substances.

When monoanhydrodihydrostrophanthidin is oxidized with perbenzoic acid in chloroform solution, 1 oxygen atom is added with the production of *oxidomonoanhydrodihydrostrophanthidin,*

[1] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **108**, 497 (1935).

[2] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **97**, 727 (1932); **99**, 693 (1932–33).

$C_{23}H_{32}O_4$. This substance yields only 2 moles of methane with Grignard's reagent and still contains the aldehyde group, since it forms an *oxime*. On further oxidation in acetone solution with permanganate, the aldehyde group is oxidized to carboxyl with formation of an acid, $C_{23}H_{32}O_7$. This acid proved to be identical in all respects with the acid obtained above by oxidation with permanganate in acetic acid. The *methyl esters* from both sources likewise showed identical properties and gave only 2 moles of methane by the Zerewitinoff method. Thus, by the action of permanganate in acetic acid, as well as by the action of perbenzoic acid, an ethylene oxide is formed on the double bond. This appears to be unusual, since we have been unable to find reference to any previous observation of the isolation of an oxide after the action of permanganate on an unsaturated substance. This reaction was not confined to the strophanthidin derivative. With both permanganate in acetic acid and perbenzoic acid, anhydro-dihydrodigitoxigenin yielded the *oxide*, $C_{23}H_{34}O_4$; *anhydrodihydro-periplogenin* yielded the *oxide*, $C_{23}H_{34}O_5$; and the dimethyl ester of anhydro-β-isostrophanthic lactone acid gave an *oxide*,[3] $C_{25}H_{34}O_8$.

Attention was next turned to the investigation of the position occupied by the oxide grouping in these substances. When an attempt was made, by the usual methods, to hydrate the oxide group to the glycol, in the case of the oxide of monoanhydrodi-hydrostrophanthidin only non-crystalline material resulted. However, after oxidation of the aldehyde group on C_{10} to carboxyl (or as its ester) or after reduction to the alcohol, it became possible to obtain crystalline products. The interpretation of these substances was complicated by the fact that in addition to the opening of the oxide group to the glycol, subsequent loss of water occurred. From the study of these substances it became apparent that the double bond which gives rise to these oxido derivatives must lie in a position different from that which leads to the formation in alkaline solution of the glycol previously described.

The oxide grouping proved to be very labile. When oxido-monoanhydrodihydrostrophanthidinic acid was dissolved in 50 per cent acetic acid, the oxide group was opened, but the glycol itself could not be obtained. The substance which was isolated proved to be an unsaturated lactone resulting from the secondary

[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **84**, 183 (1929).

loss of 2 moles of water from the glycol—one due to lactonization, as shown by the titration, and one with production of a double bond, as shown by the absorption of 1 mole of hydrogen on catalytic hydrogenation. This *anhydrodilactone*, $C_{23}H_{30}O_6$, still gave 2 moles of methane, showing the retention of two hydroxyl groups. That one of these was the secondary OH^{III} on C_3 was indicated by the formation of a *monoketone*, $C_{23}H_{28}O_6$, and by the
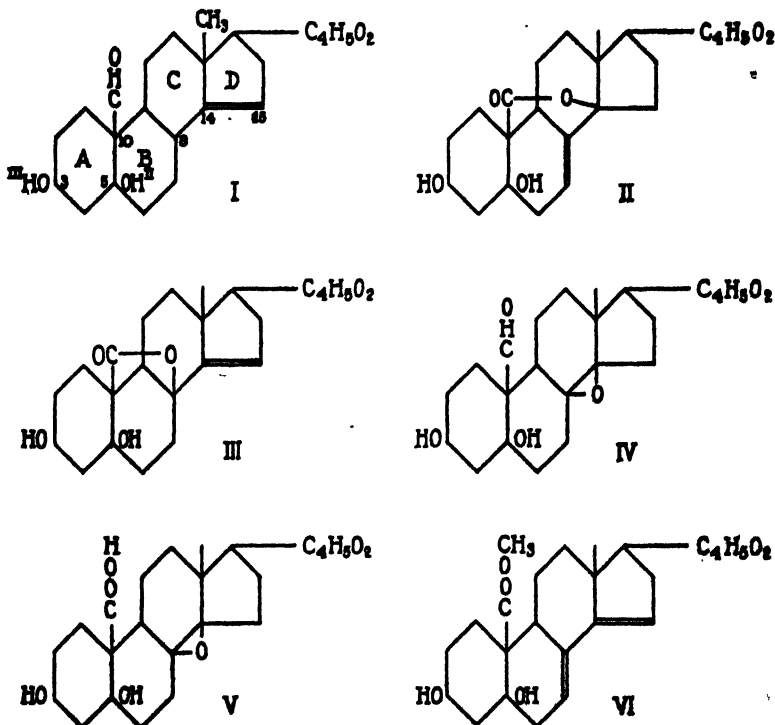
production of a *monobenzoate*, $C_{30}H_{34}O_7$. That the other hydroxyl group which was retained was the tertiary OH^{II} on C_5 was made probable by the formation with thionyl chloride of a neutral *sulfite*, $C_{23}H_{28}O_7S$. These facts thus appear to restrict the hydroxyl groups involved in the formation of the new lactone group and the double bond to those formed by hydration of the oxide group. Inasmuch as one of these hydroxyl groups must be in reactive proximity to the COOH group and the other is presum-

ably of tertiary character, in order to account for its great lability, the anhydrodilactone may be represented by either Formula II or III (the $\Delta^{8,9}$ position appears eliminated because of successful hydrogenation), oxidomonoanhydrodihydrostrophanthidin by Formula IV, and oxidodihydrostrophanthidinic acid by Formula V. The new lactone group proved to be relatively resistant to saponification, which recalls the usual behavior of the esters of the carboxyl on C_{10}. In contrast to this easy lactonization and loss
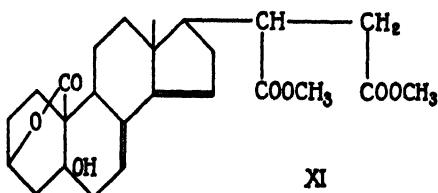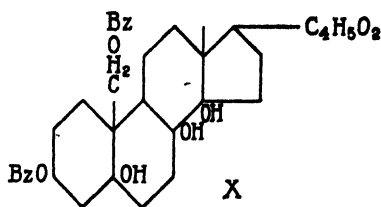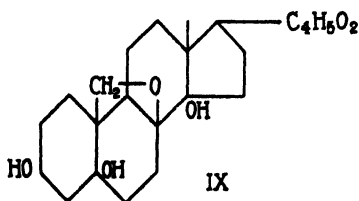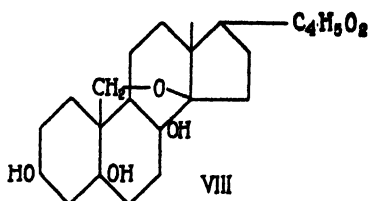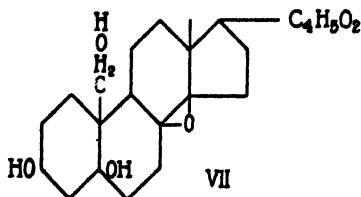
of water, the previously described glycol acid, in which the OH groups are undoubtedly on carbon atoms (14) and (15), gave no evidence of lactonization or loss of water even when treated with concentrated hydrochloric acid.

With the object of avoiding the lactonization and loss of water on opening up the oxide group, a study was made of the behavior of the *methyl ester of oxidomonoanhydrodihydrostrophanthidinic acid*. When this substance was subjected to the same mild treat-

ment with 50 per cent acetic acid, the oxido group opened as expected, but this was again followed by loss of water. A *dian-hydrolactone ester* was formed presumably with loss of both of the new hydroxyl groups and with the probable retention of OH^{II} and OH^{III}, as in the case of the anhydrodilactone. This may be represented by Formula VI.

On catalytic reduction, oxidomonoanhydrodihydrostrophan-thidin absorbed 1 mole of hydrogen. The resulting substance gave a *dibenzoate*, $C_{37}H_{42}O_8$. The conclusion is warranted that the reduction involved the aldehyde group which became a primary alcohol, and that the oxide linkage was retained. The reduction product appears, therefore, to be a *trihydroxylactone oxide*, $C_{23}H_{34}O_6$ (VII). On treatment with 50 per cent acetic acid containing a drop of H_2SO_4, an *isomeric substance* was obtained which was at first thought to have been formed by cleavage of the oxide group and loss of one of the new hydroxyl groups as water. However, the new substance gave a *monobenzoate*, $C_{30}H_{38}O_7$, and a *monoketo derivative*, $C_{23}H_{32}O_6$, on oxidation with CrO_3. These facts indicate the disappearance of one of the acylatable hydroxyls in the trihydroxylactone oxide. Active hydrogen determinations showed that in addition another hydroxyl function had been lost. The substance is therefore a *trihydroxylactone oxide*, $C_{23}H_{34}O_6$, isomeric with the substance from which it was obtained, and may be represented by either Formula VIII or IX.

If, however, a substance is used in which the primary hydroxyl group is protected, as in the case of the dibenzoate, $C_{37}H_{42}O_8$, the formation of such a new oxidic bridge should be prevented. This was found to be the case, for when the dibenzoate was subjected to the action of dilute acetic acid containing a drop of H_2SO_4, the *dibenzoate of a pentahydroxylactone*, $C_{37}H_{44}O_9$ (Formula X), resulted.

An explanation for the varying lability of the hydroxyl groups which are formed when the oxide ring is opened in each of these substances may be found in the assumption that as one goes from one derivative to another the relative *cis-trans* configurations of the hydroxyls may vary. It has been noticed many times in the course of our work on strophanthidin that changes in the molecule exert peculiar effects on other functional groups considerably

removed from the part of the molecule wherein the change has been made.   The exact cause and nature of these effects are difficult to determine.

Attempts to open the oxide group of oxidomonoanhydrodihydrodigitoxigenin and of oxidomonoanhydrodihydroperiplogenin resulted in the formation of non-crystalline products.   However, the dimethyl ester of oxido-β-isostrophanthic lactone acid yielded a *dianhydrolactone dimethyl ester*, $C_{25}H_{32}O_7$ (Formula XI), thus behaving similarly to oxidomonoanhydrodihydrostrophanthidinic ester.

The conclusion appears therefore warranted that in monoanhydrodihydrostrophanthidin, anhydrodihydroperiplogenin, and anhydrodihydrodigitoxigenin the double bond may occupy the position between C_{14} and C_{15} or C_8 and C_{14}.   Either the former may predominate in alkaline solution and the latter in neutral and acid solution, or the two forms may simultaneously occur in solution.   In the latter case, it may be that the $\Delta^{14,15}$ derivative is the form most quickly attacked by alkaline permanganate and the $\Delta^{8,14}$ that most rapidly oxidized by permanganate or perbenzoic acid in neutral or acid solution in the senses noted in the above cases.

In a recent article, Smith[4] has reported the isolation of two isomeric anhydrodigitoxigenins from digitoxigenin.   This observation explains the very early observation of Kiliani[5] of an anhydrodigitoxigenin of melting point (215–220°) higher than that reported by later workers, which varied from 183–193°.   However, Smith assumes the basis for this isomerism to be the position occupied by the double bond in each; *viz.*, $\Delta^{8,14}$ and $\Delta^{14,15}$.   If this were the case, a greater stability for such positions must be assumed than indicated by our oxidation experiments.   It appeared to us more likely that the difference between the two forms separated by Smith, β-anhydrodigitoxigenin of $[\alpha]_D^{20} = -13°$ and the α form of $[\alpha]_D^{20} = +39°$, would prove to be due to the conversion of the former into the latter by the secondary isomerizing action of the acid used for dehydration of digitoxigenin such as we have repeatedly noted in the case of the γ isomerization of numerous aglucone derivatives—for example, the conver-

[4] Smith, S., *J. Chem. Soc.*, 1050 (1935).
[5] Kiliani, H., *Arch. Pharm.*, **233**, 311 (1895); **237**, 449 (1899).

sion of strophanthidin into pseudostrophanthidin[1,6] and of α-isodigitoxigenic acid into γ-isodigitoxigenic acid.[7] In order to confirm this we have recently studied the action of hydrochloric acid on the so called β isomer of $[\alpha]_D^{22} = -14°$. When treated with the concentrated acid, it dissolves and is rapidly replaced by a sparingly soluble substance which proved to be the so called α isomer of $[\alpha]_D^{24} = +37°$ and melting point 233°. Therefore, in conformity with our earlier nomenclature, while the former should be α- the latter may be γ-anhydrodigitoxigenin.

If, as we have assumed, the oxide group of our oxido derivative lies between C_8 and C_{14} and is formed from a double bond between these carbon atoms, carbon atom (8), as formerly suggested, could not, if at all, be the only center of asymmetry involved in γ isomerization. If, however, carbon atom (8) is alone involved in such isomerization, then the conclusion would be forced that the double bond leading to oxide formation must lie between other carbon atoms, and just which, with the data at hand, would be difficult to decide.

The conversion of Smith's "β-anhydrodigitoxigenin" into the more strongly dextrorotatory form, however, may involve merely a shift of the double bond, as has been noted in the case of apocholic acid and dihydroxycholenic acid when these substances are subjected to the action of hydrogen chloride.[8]

EXPERIMENTAL

Oxidomonoanhydrodihydrostrophanthidin (Formula IV)—10 gm. of monoanhydrodihydrostrophanthidin were dissolved in 100 cc. of dry chloroform and the solution was chilled in ice. An excess of 4 per cent solution of perbenzoic acid in dry chloroform was added and the mixture was kept at 0–5° for 48 hours. The chloroform solution was washed free of acid with Na_2CO_3 and after concentration left a crystalline residue which was digested and collected with hot methyl alcohol. The substance was recrystallized from a large volume of dry acetone. It forms needles which melt at 248° and is characterized by its very sparing solubility in the usual solvents.

[6] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 795 (1927).

[7] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **88**, 199 (1930).

[8] Yamasaki, K., *Z. physiol. Chem.*, **220**, 42 (1933); **233**, 10 (1935).

C₂₃H₃₃O₆. Calculated. C 68.27,   H 7.98
           Found.   " 68.56, 68.54, " 8.19, 8.04

10.487 mg. of substance gave 1.330 cc. of CH₄ at 27° and 766.1
mm. Calculated, 2OH 8.45; found, 8.66. In this and the fol-
lowing, the results are reported as per cent hydroxyl.

Oxime of the Oxide, C₂₃H₃₂O₆—The oxime was prepared from the
above oxide with hydroxylamine hydrochloride and sodium ace-
tate in alcoholic solution. It crystallized from alcohol as prisms
and melted at 258–260° after preliminary softening.

C₂₃H₃₃O₆N. Calculated, N 3.34; found, N 3.63

Oxidomonoanhydrodihydrostrophanthidinic   Acid,   C₂₃H₃₂O₇—
1 gm. of oxidomonoanhydrodihydrostrophanthidin was suspended
in 60 cc. of dry acetone with 0.26 gm. of finely powdered KMnO₄.
The mixture was shaken for 1 hour, when all of the KMnO₄ was
used up. After removal of the solvent, the residue was thor-
oughly extracted with water. The aqueous filtrate from the
MnO₂ was carefully acidified with acetic acid and then about
half saturated with (NH₄)₂SO₄. The crystalline acid was col-
lected with a little water in which it is appreciably soluble and
was then recrystallized from dry acetone. It formed needles
which were easily soluble in moist acetone but sparingly so in
dry acetone. It softened at about 205°, then melted at 252° with
decomposition. The melting point, however, varied with differ-
ent preparations.

C₂₃H₃₂O₇. Calculated, C 66.20, H 7.92; found, C 65.68, H 7.67

The same substance was prepared directly from monoanhydro-
dihydrostrophanthidin as follows. 1 gm. of the latter was dis-
solved in 200 cc. of acetic acid. A 5 per cent aqueous KMnO₄
solution was slowly added. After addition of 30 cc., the reaction
definitely slowed up. The mixture was diluted, decolorized with
a little NaHSO₃, and then extracted with chloroform. The re-
peatedly washed chloroform extract was shaken with excess dilute
Na₂CO₃ solution, and the latter after acidification was reextracted
with chloroform. Removal of the solvent left a residue which
after recrystallization from dry acetone formed needles that
softened at 212° and decomposed at 252–254°. The melting

point showed no depression when the substance was mixed with the acid obtained from oxidomonoanhydrodihydrostrophanthidin.

Found.   C 65.86, 65.77, H 7.92, 7.79

Methyl Ester of the Acid, $C_{23}H_{32}O_7$—The ester was prepared from the acid of both sources with diazomethane. The ester from the acid prepared by direct oxidation of monoanhydrodihydrostrophanthidin formed prisms from dry acetone and exhibited a variable melting point between 229–243°.

$[\alpha]_D^{25} = +59°$ ($c = 0.635$ in pyridine)
$C_{24}H_{34}O_7$. Calculated, C 66.32, H 7.89; found, C 66.02, H 8.11

7.235 mg. of substance gave 0.852 cc. of CH_4 at 26° and 763 mm.   Calculated, 2OH 7.84; found, 7.53.

The ester of the acid prepared through the intermediate oxide was indistinguishable from the above ester. Its melting point also varied between 229–237° and showed no depression when mixed with the ester from the first source.

$[\alpha]_D^{25} = +59°$ ($c = 0.630$ in pyridine)
Found.   C 66.30, H 7.67

7.772 mg. of substance gave 0.944 cc. of CH_4 at 26° and 763 mm.   Calculated, 2OH 7.84; found, 8.28.

Anhydrodilactone, $C_{23}H_{30}O_6$ (Formula II or III)—50 mg. of oxidomonoanhydrodihydrostrophanthidinic acid were heated at 100° in 2 cc. of 50 per cent acetic acid for a few minutes. Solution was prompt and after removal of the acetic acid the anhydrodilactone crystallized. On recrystallization from acetone the substance formed sparingly soluble needles which showed a melting point which varied between 260–284°.

$[\alpha]_D^{24} = +98°$ ($c = 0.320$ in pyridine)
$C_{23}H_{30}O_6$. Calculated. C 68.62,   H 7.52
          Found.   " 68.79, 68.57, " 7.53, 7.76

The same substance was obtained also as follows. 0.5 gm. of oxidomonoanhydrodihydrostrophanthidin was dissolved in 12 cc. of pyridine by gentle warming. 15 cc. of 0.1 N NaOH were added and the transient turbidity disappeared on shaking, owing to saponification. A further 10 cc. of 0.1 N NaOH were added,

followed by 5 per cent $KMnO_4$ solution.   After 2.5 cc. of the latter had been added, a definite sharp end-point occurred.   The filtrate from the MnO_2 was acidified to litmus with HCl and the pyridine was removed on the steam bath.   The solution was then cooled and made acid to Congo red with HCl.   Immediate deposition of rosettes of needles occurred.   After recrystallization from acetone, the substance was indistinguishable from that prepared as above and the melting point of a mixture of the two showed no depression.

$$[\alpha]_D^x = +92° \ (c = 0.674 \text{ in pyridine})$$
Found.   C 68.67, 68.67, H 7.68, 7.67

12.707 mg. of substance were refluxed for 6 hours with 3 cc. of 0.1 N NaOH and then titrated back against phenolphthalein. Calculated for 2 equivalents, 0.632 cc.; found, 0.556 cc.

10.857 mg. of substance gave 1.423 cc. of CH_4 at 27° and 766.2 mm.   Calculated, 2OH 8.46; found, 8.93.

Benzoate of the Anhydrodilactone, $C_{23}H_{30}O_6$—The dilactone was acylated in pyridine solution with benzoyl chloride.   The benzoate crystallized from methyl alcohol as sparingly soluble stout prisms melting at 265°.

$C_{30}H_{34}O_7$.   Calculated, C 71.09, H 6.79; found, C 71.27, H 7.02

Sulfite of the Anhydrodilactone, $C_{23}H_{30}O_6$—50 mg. of the anhydrodilactone were chilled and covered with 1 cc. of thionyl chloride.   The substance dissolved immediately with effervescence. After standing at 0° for 30 minutes, the excess thionyl chloride was removed *in vacuo*.   The residue was dissolved in dry chloroform.   Addition of methyl alcohol caused the deposition of fine prisms which were collected with methyl alcohol and then melted at 242–243°.

$C_{23}H_{28}O_7S$.   Calculated.   C 61.57, H 6.31, S 7.14
              Found.   " 61.12, " 6.35, " 6.88

The Ketoanhydrodilactone, $C_{23}H_{28}O_6$—0.1 gm. of the anhydrodilactone, $C_{23}H_{30}O_6$, was dissolved in 5 cc. of 90 per cent acetic acid and 0.25 cc. of Kiliani's CrO_3 solution was added.   After 15 minutes the mixture was diluted and crystallization was induced by addition of $(NH_4)_2SO_4$.   The precipitate of fine needles

was collected with water. The substance melted at 251° after recrystallization from methyl alcohol.

$C_{22}H_{28}O_6$. Calculated, C 68.96, H 7.05; found, C 68.34, H 7.06

All attempts at further removal of hydroxyl groups from this substance under varying conditions resulted in the formation of non-crystalline material.

Dianhydrolactone Ester, $C_{24}H_{32}O_6$ *(Formula VI)*—The oxide group in the ester of oxidomonoanhydrodihydrostrophanthidinic acid was opened by dissolving 50 mg. in 3 cc. of 50 per cent acetic acid, heating the solution to 100°, and then adding 1 drop of 5 per cent H_2SO_4. After heating 30 seconds longer, the mixture was poured into ice and neutralized to Congo red with sodium acetate. The solution was then evaporated on the steam bath to incipient crystallization. On cooling, fine needles separated. After recrystallization from dilute acetone, the substance melted at 199° with softening at about 190°.

$C_{24}H_{32}O_6$. Calculated. C 69.37, H 7.78, OCH_3 7.46
              Found.   " 69.19, " 7.75,   "   7.22

7.545 mg. of substance gave 0.945 cc. of CH_4 at 27° and 754 mm. Calculated, 2OH 8.18; found, 8.42.

The same substance was obtained, but with greater difficulty, by omission of the sulfuric acid as used above.

The Trihydroxylactone Oxide, $C_{23}H_{34}O_6$ *(Formula VII)*—0.5 gm. of oxidomonoanhydrodihydrostrophanthidin was suspended in a large volume of alcohol and shaken with 0.25 gm. of Adams and Shriner's catalyst in an atmosphere of purified hydrogen. After about 2 days the substance completely dissolved, when approximately 1 mole of hydrogen had been absorbed. The filtrate from the catalyst was concentrated to crystallization. After recrystallization from alcohol, it formed sparingly soluble microscopic needles and melted at 265°.

$[\alpha]_D^{25} = +47°$ ($c = 0.415$ in pyridine)
$C_{23}H_{34}O_6$. Calculated. C 67.94,   H 8.43
              Found.   " 67.85, 68.20, " 8.58, 8.70

Dibenzoate of the Trihydroxylactone Oxide, $C_{33}H_{34}O_6$—The above substance was benzoylated by allowing it to stand overnight in

dry pyridine solution with excess benzoyl chloride. After the reaction mixture was poured into dilute sulfuric acid and let stand, the dibenzoate crystallized. It formed very sparingly soluble prisms from methyl alcohol, the melting point of which was not characteristic. It usually softened at about 145–150° and slowly melted on further heating.

$C_{37}H_{42}O_8$. Calculated, C 72.00, H 6.86; found, C 72.26, H 6.90

Dibenzoate of the Pentahydroxylactone, $C_{37}H_{44}O_9$ *(Formula X)*— The oxide group in the above dibenzoate was opened by heating the substance in 75 per cent acetic acid at 100° after addition of a few drops of 10 per cent H_2SO_4. After complete solution, the mixture was diluted and the amorphous precipitate was collected and recrystallized from methyl alcohol. The substance formed sparingly soluble laminated plates and melted at 210–211°.

$C_{37}H_{44}O_9$. Calculated, C 70.20, H 7.02; found, C 69.91, H 6.87

The Isomeric Trihydroxylactone Oxide, $C_{23}H_{34}O_6$ *(Formula VIII or IX)*—When the trihydroxylactone oxide ($[\alpha] = +47°$) was heated with 50 per cent acetic acid, either with or without a drop of dilute H_2SO_4, a substance isomeric with the starting material was obtained. It was recrystallized from dry acetone and formed very sparingly soluble plates melting at 282° with decomposition.

$[\alpha]_D^2 = +83°$ ($c = 0.520$ in pyridine)
$C_{23}H_{34}O_6$. Calculated. C 67.94,   H 8.43
           Found.   " 68.15, 68.41, " 8.34, 8.46

6.505 mg. of substance gave 1.175 cc. of CH_4 at 27° and 754 mm. Calculated, 3OH 12.55; found, 12.11.

Benzoate of the Trihydroxylactone Oxide ($[\alpha]_D = +83°$)—Upon acylation with benzoyl chloride in pyridine solution, the preceding substance, in contrast to its isomer of $[\alpha]_D = +47°$, gave a monobenzoate. After recrystallization from methyl alcohol, it formed plates and melted at 274–275°.

$C_{30}H_{38}O_7$. Calculated, C 70.55, H 7.50; found, C 70.41, H 7.73

The Ketodihydroxylactone Oxide, $C_{23}H_{32}O_6$—0.2 gm. of the oxide ($[\alpha]_D = +83°$) was dissolved in 10 cc. of 90 per cent acetic acid. 0.3 cc. of 20 per cent aqueous CrO_3 solution was added and after

standing 45 minutes the mixture was diluted and extracted with chloroform. After removal of the solvent the resulting substance was recrystallized from methyl alcohol. It formed sparingly soluble platelets and melted at 272° with decomposition after softening at about 260°. The analytical figures for carbon were slightly high. This was doubtlessly caused by partial loss of the OH^{II} which is β to the CO group.

$C_{23}H_{32}O_6$. Calculated, C 68.27, H 7.98; found, C 68.85, H 7.82

All attempts to remove the reactive hydroxyl with the formation of an unsaturated ketone resulted in the formation of noncrystalline material.

The Dimethyl Ester of Oxido-β-Isostrophanthic Lactone Acid— 0.2 gm. of the dimethyl ester of anhydro-β-isostrophanthic lactone acid was dissolved in 5 cc. of chloroform and treated with an excess of a 4 per cent chloroform solution of perbenzoic acid. After standing 48 hours at 0° the solution was washed free of acid with Na_2CO_3. Evaporation of the chloroform solution left a residue which was recrystallized from dry acetone. The substance formed sparingly soluble needles and melted at 244–245° after softening at 240°.

$[\alpha]_D^{20} = -26°$ ($c = 0.510$ in pyridine)
$C_{23}H_{34}O_8$. Calculated, C 64.90, H 7.41; found, C 64.79, H 7.44

The same substance resulted on oxidation of the dimethyl ester with $KMnO_4$ in acetic acid as in other cases. The material thus obtained proved to be indistinguishable from that made by the perbenzoic acid method. It melted at 244–245° after softening at 240° and showed no depression when mixed with material made by the other method.

$[\alpha]_D^{20} = -24°$ ($c = 0.685$ in pyridine)
Found. C 65.30, H 7.65

12.870 mg. of substance gave 0.632 cc. of CH_4 at 27° and 751.3 mm. Calculated, 1OH 3.67; found, 3.28.

The Dianhydrolactone Dimethyl Ester, $C_{25}H_{32}O_7$ (Formula XI)— A suspension of 50 mg. of the above oxide in 3 cc. of 50 per cent acetic acid, heated on the steam bath, was treated with 2 drops of 10 per cent H_2SO_4. The substance promptly dissolved. After

neutralisation to Congo red with sodium acetate, the solution was evaporated to copious crystallization. The substance was recrystallized from dilute acetone and formed needles which melted at 178–180°.

$C_{25}H_{32}O_7$. Calculated. C 67.53, H 7.26, OCH$_3$ 13.96
Found. " 67.22, " 7.66, " 13.68

10.830 mg. of substance gave 0.725 cc. of CH$_4$ at 28° and 754 mm. Calculated, 1OH 3.84; found, 4.48.

Oxidomonoanhydrodihydrodigitoxigenin—This was prepared by both of the methods used in the other cases. After recrystallization from acetone-ether the substance formed diamond-shaped platelets which softened at 205–210° and cleared at 215°. The melting point varied considerably with different preparations.

$C_{23}H_{34}O_4$. Calculated, C 73.74, H 9.16; found, C 73.95, H 9.11

All attempts to open the oxide in this substance resulted in the formation of non-crystalline products.

Monoanhydrodihydroperiplogenin—2.5 gm. of dihydroperiplogenin were dissolved in a mixture of 125 cc. of methyl alcohol and 25 cc. of water. 100 cc. of HCl (1.10) were then added and the mixture was allowed to stand overnight at room temperature. After dilution the crystalline precipitate was collected and recrystallized from acetone. The substance formed irregular leaflets which melted at 230–234° after preliminary softening.

$C_{23}H_{34}O_4$. Calculated. C 73.74, H 9.16
Found. " 74.01, 73.90, " 9.30, 9.16

Oxidomonoanhydrodihydroperiplogenin—This was prepared by both the perbenzoic acid and KMnO$_4$ methods. The substance was recrystallized from acetone and formed sparingly soluble prisms which melted at 252–258°. For the substance prepared with perbenzoic acid

$[\alpha]_D^{20} = +54°$ (c = 0.435 in pyridine)
$C_{23}H_{34}O_5$. Calculated, C 70.59, H 8.72; found, C 70.78, H 8.78

For that prepared by the use of KMnO$_4$ in acetic acid

$[\alpha]_D^{20} = +51°$ (c = 0.460 in pyridine)
Found. C 70.67, H 8.88

Attempts to open the oxide in this substance failed to yield any crystalline product.

STROPHANTHIN

XXXIV. CYANHYDRIN SYNTHESES WITH DIHYDROSTRO-PHANTHIDIN AND DERIVATIVES

By WALTER A. JACOBS and ROBERT C. ELDERFIELD

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

It has been a repeated experience with the derivatives of strophanthidin that the aldehyde group, as such, on carbon atom (10), or after oxidation to carboxyl, may react with OH^I and OH^{III} to give lactols or lactones, provided the proper steric arrangement is present. However, in no case has there been evidence that OH^{II} can react in this way—a fact which is readily explained, since its position on carbon atom (5) is now known to be β to the groups on carbon atom (10).

At one stage of the investigation of the structure of strophanthidin it was of interest to study the effect of the addition of 1 carbon atom by means of the cyanhydrin synthesis on the ability of the new carboxyl group thus formed to interact with the various hydroxyl groups of this aglucone and so to obtain additional evidence as to their relative positions.

Although strophanthidin itself proved unsuited, dihydrostrophanthidin (I) was found to yield very readily an intermediate cyanhydrin (II) which was not isolated, as such, but was found to be readily hydrolyzed by dilute acetic acid. From the resulting mixture two isomeric, non-nitrogenous substances were obtained which were found to be neutral and, from the analysis, to possess the formula $C_{24}H_{34}O_7$. Investigation showed that these substances must be what might be loosely called α- and β-homodilactones (III and IV) in which the isomerism is due to the new center of asymmetry established at the carbon atom previously contained in the aldehyde group of dihydrostrophanthidin and now carrying a new hydroxyl group. At first it was concluded,

on the basis of our older structure for strophanthidin, that the new lactonization occurred on OH^{III}. Both isomers readily yielded anhydro derivatives like dihydrostrophanthidin itself, respectively α- and β-*anhydrohomodilactones* (V and VI), which appeared to exclude OH^I as that involved in the new lactone
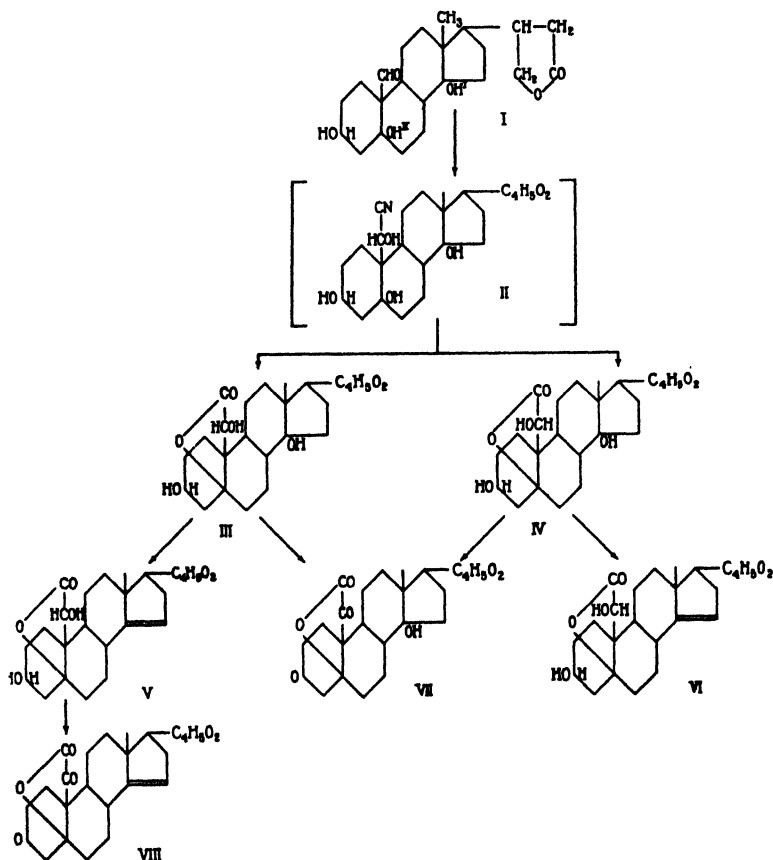
group.   The α-homodilactone (no crystalline derivative could be obtained from the β derivative) yielded a *monobenzoate* which, however, could still be explained by acylation of the new hydroxyl group.   The benzoate in turn could be readily dehydrated to the *benzoate of the α-anhydrohomodilactone* with loss of OH^I.

However, according to the new formula for strophanthidin, lactonization on OH^{III} would make the new lactone group an ϵ-lactone and it appeared therefore possible that OH^{II} on C_5 might be the hydroxyl group really involved, since it would now become γ to the new carboxyl group. This has more recently been found to be the case and was demonstrated in the following way. When the α-dilactone was oxidized with chromic acid, a substance, $C_{24}H_{30}O_7$, was obtained which was shown to be a *diketohomodilactone* (VII). This could have been formed only if both OH^{III} and the new hydroxyl group were free. Similarly, the α-anhydrohomodilactone gave a substance $C_{24}H_{28}O_6$ and, therefore, an *anhydrodiketohomodilactone* (VIII). Oxidation of the β-dilactone gave a substance which proved to be identical with that obtained above from the α isomer, a fact which confirms the view that the isomerism of the two substances is due to the asymmetry of the new hydroxyl-bearing carbon atom and which is therefore lost on oxidation to carbonyl.

At one stage of the work we had occasion also to attempt the cyanhydrin synthesis with the benzoate of dihydrostrophanthidin. In this case, the sparingly soluble intermediate cyanhydrin, $C_{31}H_{39}O_7N$, was obtained without difficulty. This again was readily hydrolyzed to form the benzoate of the homodilactone, $C_{31}H_{38}O_8$, which proved to be isomeric with that obtained above by benzoylation of the α-dilactone. We were unable to determine whether the former might be the benzoate of the β-dilactone, since the latter on acylation gave material which was difficult to purify and suggested further acylation. The above benzoate was readily converted into an *anhydrohomodilactone benzoate* with loss of OH^I.

<div align="center">EXPERIMENTAL</div>

The α- and β-Homodilactones, $C_{24}H_{34}O_7$ (III and IV)—10 gm. of dihydrostrophanthidin were dissolved in 50 cc. of alcohol, and to the solution 2.5 gm. of KCN in 5 cc. of water and 2.5 gm. of NH_4Cl in 10 cc. of water were added. After standing overnight, the mixture was made strongly acid with acetic acid and evaporated to copious crystallization on the steam bath. The collected material was dissolved in hot 85 per cent alcohol and allowed to crystallize. The substance thus obtained formed needles and

melted at 254° with effervescence. This isomer will be designated the α-homodilactone, and derivatives of it prefixed by α-.

$[\alpha]_D^{25}$ = −36° (c = 1.0 in pyridine)
$C_{24}H_{34}O_7$.   Calculated.   C 66.32,   H 7.89
           Found.   " 66.10, 66.19, " 7.77, 8.23

The titration of this substance after saponification with alkali offered difficulties. Under different conditions of saponification, the results obtained always lay between 1 and 2 equivalents. The failure to obtain quantitative figures for the second lactone group was perhaps due partly to ease of relactonization and perhaps partial steric rearrangement which affected the latter, or in part to resistance to saponification.

Thus, 0.1039 gm. of substance was boiled with 14 cc. of 0.1 N NaOH for 3 hours and titrated back against phenolphthalein. 3.50 cc. of 0.1 N NaOH were consumed. Calculated for 2 equivalents, 4.78 cc. In a similar experiment 0.1029 gm. required 0.41 cc. of N NaOH. Calculated for 2 equivalents, 0.47 cc.

The mother liquor from the first fraction was concentrated and, after removal of an additional crop of the above substance, a second isomer, which was considerably more soluble, was obtained. After recrystallization from 30 per cent alcohol it formed stout prisms which softened to a paste at about 175° and melted at 235° with effervescence. The melting point varied greatly with the rate of heating. This is the β-homodilactone and its derivatives will be prefixed by β-.

$[\alpha]_D^{25}$ = +30° (c = 0.567 in pyridine)
           Found. C 66.68, 66.38, H 7.81, 7.97

Difficulty was also experienced with the titration of this isomer. Thus, 0.1123 gm. of substance after refluxing 4 hours with 0.1 N NaOH consumed 3.00 cc. Calculated for 2 equivalents, 5.18 cc.

Again, 0.0899 gm. after refluxing for 5½ hours with 3 cc. of N NaOH consumed 0.34 cc. of N alkali. Calculated for 2 equivalents, 0.414 cc.

The α- and β-Anhydrohomodilactones (V and VI)—The dehydration of the above lactones was performed in each case by dissolving in a sufficient quantity of hot 50 per cent alcohol and then making the solutions distinctly acid with HCl. On heating

for about 15 minutes, copious crystallization began. After cooling, the anhydrolactones were collected and recrystallized from a large volume of alcohol.

The α-anhydro derivative formed needles which melted at 302–303°.

$[\alpha]_D^{24} = -72°$ ($c = 1.03$ in pyridine)
$C_{24}H_{32}O_6$. Calculated. C 69.19,   H 7.75
                   Found.   " 69.18, 69.29, " 7.86, 7.86

The β-anhydrodilactone formed rectangular plates which melted at 305° with effervescence after preliminary softening.

$[\alpha]_D^{27} = -5°$ ($c = 0.960$ in pyridine)
                   Found. C 69.43, 69.45, H 8.26, 8.02

Benzoate of the α-Homodilactone—This was prepared in the usual manner by acylation with benzoyl chloride in dry pyridine. The benzoate was recrystallized from methyl alcohol and formed needles which melted at 320°.

$C_{31}H_{38}O_8$. Calculated, C 69.11, H 7.12; found, C 69.27, H 7.25

The Diketohomodilactone (*VII*)—0.3 gm. of the α-lactone was dissolved in 15 cc. of 90 per cent acetic acid and 1 cc. of Kiliani's CrO_3 reagent was added. Oxidation was prompt and after 15 minutes the mixture was diluted and extracted with ethyl acetate. The washed and dried ethyl acetate solution on concentration gave the diketone which was recrystallized from a mixture of acetone and ether. It formed hexagonal plates and melted at 189–190° with decomposition.

$[\alpha]_D^{27} = +73°$ ($c = 0.775$ in pyridine)
$C_{24}H_{30}O_7$. Calculated. C 66.94,   H 7.03
                   Found.   " 66.96, 66.95, " 7.14, 7.16

When the β-lactone was oxidized in the same manner, the resulting diketone was indistinguishable from the above substance. It melted also at 189–190° and showed no depression when mixed with the diketone from the α-lactone.

$[\alpha]_D^{27} = +75°$ ($c = 0.715$ in pyridine)
                   Found. C 67.02, H 7.14

Anhydrodiketohomodilactone (VIII)—60 mg. of the anhydro-α-homodilactone were dissolved in 5 cc. of 90 per cent acetic acid and 0.5 cc. of Kiliani's CrO_3 solution was added.   After 15 minutes the solution was diluted and the crystalline precipitate was collected.   After recrystallization from dilute acetone the substance formed diamond-shaped leaflets which melted at 196° with effervescence after softening at about 191°.

$C_{24}H_{28}O_6$.   Calculated, C 69.87, H 6.85; found, C 69.77, H 6.65

Cyanhydrin of Dihydrostrophanthidin Benzoate—0.3 gm. of dihydrostrophanthidin benzoate was dissolved in 5 cc. of pyridine. A solution of 0.17 gm. of KCN in 2 cc. of water was added, followed by 2.5 cc. of 10 per cent NH_4Cl solution.   On standing overnight, a crystalline deposit formed which was collected with water.   The cyanhydrin was recrystallized from acetone and melted at 258° with effervescence.

$C_{31}H_{39}O_7N$.   Calculated.   C 69.23, H 7.32, N 2.66
Found.   " 69.27, " 7.25, " 2.63

The Homodilactone Benzoate—The cyanhydrin was hydrolyzed by repeated evaporation on the steam bath with 50 per cent acetic acid.   The neutral benzoate of the homodilactone was collected with water and recrystallized from alcohol.   It formed glistening prisms and melted at 305–306° with effervescence.

$C_{31}H_{38}O_8$.   Calculated, C 69.11, H 7.12; found, C 69.30, H 7.00

The Anhydrohomodilactone Benzoate—0.1 gm. of the homodilactone benzoate, $C_{31}H_{38}O_8$, was dissolved in 25 cc. of 85 per cent alcohol containing 2.5 cc. of HCl (1.19) and the solution was refluxed for 15 minutes.   On dilution, minute needles separated. The anhydro compound after recrystallization from alcohol melted at 265–268° after slight preliminary softening.

$[\alpha]_D^{25} = -38°$ ($c = 0.745$ in pyridine)
$C_{31}H_{36}O_7$.   Calculated, C 71.50, H 6.98; found, C 71.18, H 6.95

STROPHANTHIN

XXXV. THE NATURE OF "THE ACID, $C_{23}H_{30}O_8$," FROM STROPHANTHIDIN

By ROBERT C. ELDERFIELD

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When strophanthidinic acid (I) was oxidized after saponifica-· tion in alkaline solution with $KMnO_4$, a new acid was obtained.[1] From its behavior on titration and from the analytical data, this acid was assumed to be dibasic and was assigned the formula $C_{23}H_{30}O_8$. At the same time, the presence of a carbonyl group as well as retention of the original secondary hydroxyl group (OH^{III}) was noted. However, when the acid was boiled with alkali, a new acid, provisionally assigned the formula, $C_{23}H_{32}O_9$, was formed, apparently by addition of the elements of water. No tendency to relactonize was noted in this acid. The behavior of strophanthidinic acid on oxidation thus remained distinct from that of other strophanthidin derivatives, and the exact nature of the acid $C_{23}H_{30}O_8$ remained obscure.

Recently it has become possible to clarify the chemistry of these substances. Reexamination of the methyl ester of the acid $C_{23}H_{30}O_8$ has shown that, contrary to the earlier belief, it is not a dimethyl ester, but a monomethyl ester. The acid must therefore be monobasic. This fact gave the clue to its proper interpretation. The consumption of a second equivalent of alkali, which was noted on direct titration, must now be interpreted as due to the presence of an easily opened lactone group. At the same time, the methoxyl figures obtained with the monomethyl ester indicated a smaller molecular weight than that originally assumed.

[1] Jacobs, W. A., *J. Biol. Chem.*, **57**, 553 (1923).

It became therefore necessary to attempt a reinterpretation of the previously obtained analytical figures with all of the substances of this series and also to prepare several new derivatives the analyses of which would afford criteria for this purpose. This study led to the conclusion that the lactone acid was a product of partial degradation of the molecule and that it possesses, not a formula $C_{22}H_{30}O_8$, but that of $C_{21}H_{28}O_7$. This was confirmed by the preparation and analysis of the *p-bromophenylhydrazone* of the monomethyl ester and of the *diketone*, $C_{22}H_{28}O_7$, formed from the ester by oxidation of OH^{III}, and of its dioxime, $C_{22}H_{30}O_7N_2$. The formation of the acid "$C_{23}H_{32}O_9$" would then be explained
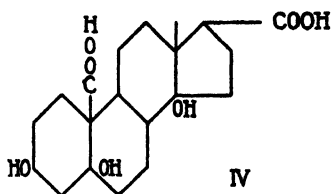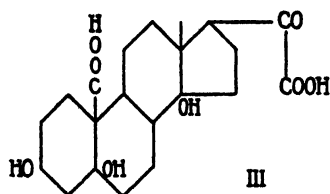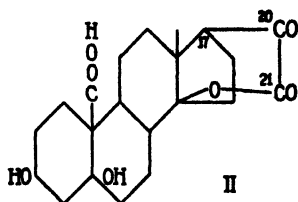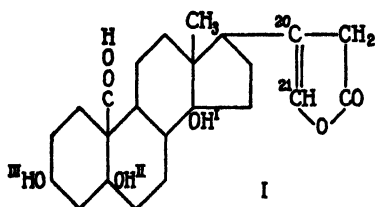
by opening of the lactone group with addition of the elements of water, and it should yield a dimethyl ester. This was found to be the case, and the formula of the dibasic acid therefore must be revised to $C_{21}H_{30}O_8$.

The smaller formula having been established, it at once appeared obvious that the only way in which degradation of the molecule of strophanthidinic acid by loss of C_2H_2 could occur was by shortening of the side chain. Loss of 2 carbon atoms and oxidation of the aldehyde group of C_{21} to carboxyl followed by lactonization on OH^I would account for the formation of the acid $C_{21}H_{28}O_7$. This would then be an α-ketolactone acid as represented by Formula II. On treatment with alkali, the lactone

opens and the resulting acid, $C_{21}H_{30}O_8$, may be represented by Formula III. Under the influence of alkali, the fragment of the side chain on C_{17} can rearrange at this point by enolization of the carbonyl group so that it now becomes *trans* to OH^I, an arrangement which would prevent relactonization.

This interpretation was confirmed by a study of the reactions of the acid $C_{21}H_{30}O_8$. The latter with *o*-phenylenediamine gave

TABLE I

Reinterpretation of Previous Analytical Data

Substance	Formula	Calculated	Found
Ketolactone acid (II)	$C_{21}H_{28}O_7$	C 64.25, H 7.19	C 63.69, H 6.94 " 63.71, " 6.94
Methyl ester	$C_{22}H_{30}O_7$	" 64.99, " 7.44, OCH_3 7.63	" 64.84, " 7.27 " 64.79, " 7.15, OCH_3 7.83
Benzoate of methyl ester	$C_{29}H_{34}O_8$	C 68.20, H 6.72, OCH_3 6.08	C 67.96, H 6.57, OCH_3 6.06
Phenylhydrazone of methyl ester	$C_{28}H_{36}O_6N_2$	C 67.55, H 7.30, N 5.63	C 67.58, H 7.27, N 5.80
Oxime of methyl ester	$C_{22}H_{31}O_7N$	C 62.67, H 7.42	C 62.97, H 7.40
Dibasic keto acid (III)	$C_{21}H_{30}O_8$	" 61.43, " 7.37	" 61.63, " 7.02 " 61.75, " 7.15
Dimethyl ester	$C_{23}H_{34}O_8$	C 62.98, H 7.82	C 63.06, H 7.72 " 63.06, " 7.69
Benzoate of di-methyl ester	$C_{30}H_{38}O_9$	C 66.38, H 7.06, OCH_3 11.43	C 66.33, H 6.99, OCH_3 11.50
Oxime of dimethyl ester	$C_{23}H_{35}O_8N$	C 60.89, H 7.78	C 60.38, H 7.69

a *quinoxaline*, $C_{27}H_{34}O_6N_2$. When the acid was oxidized with hydrogen peroxide,[2] further degradation occurred with the formation of an *acid*, $C_{20}H_{30}O_7$ (IV), which was characterized by its *dimethyl ester*, $C_{22}H_{34}O_7$. These reactions are characteristic of α-keto acids.

In the earlier report of the formation of these acids, the analytical data presented certain discrepancies. When transferred to

[2] Holleman, A. F., *Rec. trav. chim. Pays-Bas*, **23**, 169 (1904).

the newer formulations, it will be seen that they are in better agreement with the requirements.   These recalculations together with data more recently obtained are shown in Table I.

EXPERIMENTAL

The Diketone, $C_{22}H_{28}O_7$—0.1 gm. of the methyl ester of the acid $C_{21}H_{28}O_7$ was dissolved in 5 cc. of 90 per cent acetic acid. 0.2 cc. of Kiliani's CrO_3 solution was added, and after standing 30 minutes the solution was diluted and the ketone was salted out with $(NH_4)_2SO_4$. It was recrystallized from acetone-ether and formed rhombs which melted at 228°.

$C_{22}H_{28}O_7$. Calculated, C 65.31, H 6.98; found, C 65.64, H 6.87

Dioxime of the Diketone, $C_{22}H_{28}O_7$—This was prepared in the usual manner.   On recrystallization from dilute methyl alcohol it formed needles and melted at 233° with decomposition after sintering at about 210°.

$C_{22}H_{30}O_7N_2$. Calculated, N 6.45; found, N 6.62

The Acid, $C_{20}H_{30}O_7$ *(IV)*—0.2 gm. of the acid $C_{21}H_{30}O_8$ was dissolved in 20 cc. of water.   2 cc. of 30 per cent hydrogen peroxide solution were added and the mixture was allowed to stand 48 hours, during which evolution of CO_2 took place.   During the reaction a substance crystallized as stout wedges.   These were collected and recrystallized from dry acetone.   The acid melted at 295° with decomposition and showed no appreciable optical rotation.

$C_{20}H_{30}O_7$. Calculated. C 62.79,   H 7.91
           Found.   " 62.81, 62.73, " 7.99, 7.91

Dimethyl Ester of the Acid, $C_{20}H_{30}O_7$—This was prepared with diazomethane.   It formed prisms from acetone-ether, which melted at 200°.

$C_{22}H_{34}O_7$. Calculated. C 64.35, H 8.35, OCH_3 15.11
           Found.   " 64.47, " 8.08,   "   14.96

p-Bromophenylhydrazone of the Methyl Ester, $C_{22}H_{30}O_7$—0.2 gm. of the methyl ester and 0.15 gm. of *p*-bromophenylhydrazine were heated in 10 cc. of acetic acid at 100° for 30 minutes.   Deposition

of the hydrazone began after a few minutes. The substance was recrystallized from ethyl acetate and formed fine needles which melted at 273°.

$C_{28}H_{35}O_6N_2Br$.   Calculated.   C 58.41, H 6.13, N 4.87, Br 13.89
                   Found.   " 58.59, " 6.17, " 5.14, " 14.29

The Quinoxaline, $C_{27}H_{34}O_6N_2$—0.1 gm. of the acid, $C_{21}H_{30}O_6$, was dissolved in 8 cc. of hot water. 44 mg. of *o*-phenylenediamine were added and the solution was heated 30 minutes at 100°. The amorphous precipitate was collected with a little water and recrystallized from ethyl acetate. The quinoxaline formed stout prisms and melted at 222–224° with decomposition.

$C_{27}H_{34}O_6N_2$.   Calculated, N 5.81; found, N 5.55

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XLIII. THE COMPOSITION OF LEPROSIN*

By R. J. ANDERSON, J. A. CROWDER,† M. S. NEWMAN,‡ AND F. H. STODOLA§

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In the purification of the phosphatide fraction of the leprosy bacillus as described by Uyei and Anderson (1) a notable amount of a neutral wax-like substance was obtained from the ether-acetone mother liquors. The present report deals with the chemical composition of this substance which will be designated by the name leprosin. After suitable purification leprosin was obtained as a voluminous, snow-white, amorphous powder containing only carbon, hydrogen, and oxygen. The substance was optically active and melted at about 50°.

The cleavage products obtained on saponification consisted of a series of ordinary fatty acids, from myristic acid to tetracosanic acid, a new hydroxy acid which will be designated by the name leprosinic acid, glycerol, and two aliphatic secondary alcohols. Leprosin is therefore a mixture of solid glycerides and waxes.

One of the alcohols was identified as *d*-eicosanol-2 and in all probability the other alcohol was *d*-octadecanol-2. The same alcohols have been previously identified as constituents of the so called wax fraction of the timothy bacillus by Pangborn and Anderson (2).

Although the substance called leprosinic acid has not been ob-

tained in crystalline form nor in a definitely homogeneous state, yet some description of its properties seems justified at this time because it represents the largest component of leprosin. The substance is acid-fast and it is optically active, $[\alpha]_D = +4.0°$. In composition it corresponds approximately to the formula $C_{44}H_{88}O_3$, but the values for molecular weight are frequently at variance with this formula, usually being much higher. This phenomenon may depend upon partial lactone formation. Leprosinic acid has an iodine number of 6 (Hanus) but in chloroform solution it absorbs bromine slowly, giving a bromine derivative which contains approximately 2 atoms of bromine.

Neither leprosin nor any of its cleavage products gave any sterol color reactions; hence we may conclude that the substance does not contain sterols.

The so called wax fractions of the acid-fast bacteria which we have examined all contain large amounts of hydroxy acids of very high molecular weight. The principal constituent of the wax from the human tubercle bacillus which was designated by the term "unsaponifiable wax" (3) was a hydroxy acid. An analogous acid was also isolated from the timothy bacillus wax (2). The properties and solubilities of these acids are quite similar, but some are optically active, while others are inactive (3). All of these acids are acid-fast. The elucidation of the structure of these interesting compounds will remain a problem for future study.

<div align="center">EXPERIMENTAL[1]</div>

The crude leprosin used in the present investigation was a pink-colored powder which had been isolated from the mother liquors in the purification of *Bacillus lepræ* phosphatide as described in Paper XXVI of this series (1). The substance was dissolved in ether and the solution was treated with norit until nearly colorless. The ethereal solution was concentrated to about 300 cc. and mixed with an equal volume of acetone. On cooling the solution in ice water, a nearly white amorphous precipitate separated. After five reprecipitations a snow-white voluminous powder consisting of fine globular particles was obtained. A total of about 75 gm. of purified leprosin was obtained, m.p. 50–51°,

[1] We are indebted to Dr. A. J. McAmis for carrying out the purification of the first portion of leprosin.

$[\alpha]_D$ in $CHCl_3 = +4°$.   The iodine number (Hanus) was 5.   The substance was neutral in reaction and it was free from ash, phosphorus, nitrogen, and sulfur.   Analysis: found, C 79.69, H 13.29.

Saponification of Leprosin and Separation of the Cleavage Products

The leprosin was saponified by refluxing 10 to 20 gm. portions with an excess of alcoholic potassium hydroxide for about 4 hours. The substance melted in the hot alcohol but dissolved gradually, giving a faintly cloudy solution.   As the solution cooled, a precipitate separated but it dissolved on warming.   About one-half of the alcohol was distilled off and the solution was diluted with an equal volume of water, whereupon a cloudiness appeared which cleared up on warming.   On standing at room temperature, a dense precipitate separated which was filtered off, washed with cold dilute alcohol, and dried *in vacuo*.   The dried precipitate amounted to about 70 per cent of the leprosin.   Some 70 gm. of leprosin were saponified.

Unsaponifiable Matter

The filtrate and washings from the above precipitate were diluted with more water and extracted three times with ether. The ethereal extract was washed with water, dried with sodium sulfate, filtered, and the ether was distilled off.   A mere trace of a residue was obtained; hence one might conclude that leprosin did not contain any unsaponifiable matter.   However, as will be shown later, the unsaponifiable matter consisted of higher alcohols which were precipitated from the dilute alcoholic solution along with insoluble potassium salts of higher acids.

Separation of Fatty Acids—The alkaline solution after extraction with ether was acidified and the fatty acids were extracted with ether and recovered in the usual manner.   The fatty acids formed a white solid and amounted to about 30 per cent of the leprosin.

Examination of Water-Soluble Constituents

Isolation of Glycerol—The acidified aqueous solution, after the fatty acids had been extracted, was neutralized with potassium hydroxide and evaporated to dryness under reduced pressure. The residue was extracted several times with absolute alcohol.

The alcoholic extract on evaporation to dryness yielded a thick syrup. A portion of the syrup when heated with acid potassium sulfate gave a strong odor of acrolein. Another portion of the syrup was benzoylated by the method of Einhorn and Hollandt (4). Crystalline glyceryl tribenzoate was isolated in nearly quantitative yield from the reaction mixture. The substance melted at 75–76° and gave no depression when mixed with a sample of glyceryl tribenzoate. The amount of crude glycerol obtained was from 6 to 7 per cent of the leprosin.

A portion of the crude syrup after being boiled for some time with dilute acid and neutralized did not reduce Fehling's solution, thus indicating the absence of carbohydrate.

TABLE I

Fatty Acids from Leprosin

Fraction No.	Weight of esters	M.p.		Free acids	
				M.p.	Mol wt.
	gm.	°C.		°C.	
1	0.9	17–18	n_D^{25} 1.4345	53–54	232
2	1.7	28–29	n_D^{5} 1.4270	62–63	257
3	4.0	37–38	" 1.4310	70–71	284
4	1.8	48–49	" 1.4380	77–78	352
5	0.6	Residue in distilling flask		81–82	363

Examination of Fatty Acids—A portion of the mixed acids, weighing 17.1 gm., on methylation yielded 17.8 gm. of esters. The latter were fractionated repeatedly until apparently pure fractions were obtained. Owing to the presence of at least five different acids the separation into pure components was a slow and tedious operation and only small amounts of pure fractions were obtained. The purified esters were saponified and the free acids were isolated and recrystallized, the first member from aqueous alcohol and the others from acetone. The results are given in Table I.

Fraction 1 represents slightly impure myristic acid. A sample of the acid mixed with myristic acid, m.p. 56°, gave a depression of 1°. The slightly high molecular weight likewise indicates an admixture with a higher acid such as palmitic acid. Fraction 2

was pure palmitic acid and there was no depression of the melting point when mixed with palmitic acid.   Fraction 3 was pure stearic acid as shown by its properties and a mixed melting point.   Fraction 4 was probably not pure.   The molecular weight corresponds to that of a saturated C_{23} acid.   The melting point of this acid was 77–78° after one crystallization and could not be changed by eight recrystallizations from acetone.   Fraction 5 is apparently a tetracosanic acid, $C_{24}H_{48}O_2$.   All of the free acids crystallized in the form of colorless plates.

Examination of the Insoluble Potassium Salts

The material which precipitated from the saponification mixture, as mentioned above, was believed at first to consist of potassium salts of higher fatty acids.   It became apparent, however, in attempting to purify this material that it was a mixture containing neutral substances.   The separation of the acidic from the neutral substances was accomplished in the following manner. The total material was freed from potassium by shaking in a separatory funnel with ether and dilute hydrochloric acid, during which operation everything went into solution.   The ethereal extract was washed with water, dried with sodium sulfate, filtered, and evaporated to dryness.   The residue was dissolved in a large volume of hot alcohol and an alcoholic solution of lead acetate was added in excess.   A copious precipitate of lead salts separated immediately and increased on cooling to room temperature.   The lead salts were filtered off, washed with cold alcohol, and examined, as will be described later.

Isolation of Neutral Material

The alcoholic filtrate and washings from the lead salts were concentrated nearly to dryness under reduced pressure.   The residue was mixed with water, and the insoluble portion was filtered off and washed with water, after which it was dissolved in ether and the solution was washed with water containing a little hydrochloric acid.   The ethereal solution was washed with water until free from acid, dried with sodium sulfate, filtered, and the ether was distilled off.   The residue was a colorless oil when warm and a white crystalline solid at room temperature.   This neutral fraction amounted to 7 per cent of the leprosin.

Examination of Neutral Substance. Isolation of d-Eicosanol-2

The neutral substance was readily soluble in the ordinary organic solvents on warming. On cooling these solutions, colorless globular particles were usually obtained, but from ethyl acetate the substance separated in massive clusters of colorless prismatic crystals. A quantity of the neutral material weighing 2.4 gm., on repeated recrystallization from ethyl acetate, gave 1.4 gm. of a product having the constant melting point of 63–64°.

Analysis—11.21 mg., 10.12 mg. substance gave 14.18 mg., 12.90 mg. H_2O and 32.98 mg., 29.70 mg. CO_2; mol. wt. in camphor 293, 301

$C_{20}H_{42}O$ (298). Calculated. C 80.53, H 14.09
Found. " 80.23, 80.04, " 14.15, 14.26

Rotation—0.3610 gm. of substance dissolved in ether and diluted to 10 cc. gave in a 1 dm. tube an average reading of +0.25°; hence $[\alpha]_D^{21} = +6.92°$.

The properties and composition of this substance correspond to those of *d*-eicosanol-2 which was isolated from the timothy bacillus wax (2). A mixed melting point with *d*-eicosanol-2, m.p. 63°, gave no depression. The optical rotation of *d*-eicosanol-2, which was reported as +4° in chloroform, was redetermined in ethereal solution and was found to be +6.93°. The identification of the substance as *d*-eicosanol-2 was further confirmed by oxidizing the alcohol to the corresponding ketone.

Oxidation of d-Eicosanol-2 to 2-Eicosanone

The alcohol, 0.36 gm., was oxidized in glacial acetic acid solution with chromic acid, and the oxidation product was isolated and crystallized from methyl alcohol as described in a former paper (2). The ketone, which crystallized in large colorless plates, weighed 0.3 gm. and it melted at 60–61°. A mixed melting point with 2-eicosanone prepared from the timothy wax alcohol showed no depression.

Analysis—10.76 mg. substance gave 13.115 mg. H_2O and 31.88 mg. CO_2
$C_{20}H_{40}O$ (296). Calculated. C 81.08, H 13.51
Found. " 80.81, " 13.64

Semicarbazone—The semicarbazone was prepared from 0.1 gm. of the ketone and recrystallized from alcohol. The yield was

practically quantitative. The semicarbazone crystallized in long thin colorless plates and melted at 130–131°. A mixed melting point with the semicarbazone prepared from the timothy wax 2-eicosanone gave no depression.

Analysis—4.065 mg., 4.041 mg. substance gave 4.40 mg., 4.34 mg. H_2O and 10.63 mg., 10.60 mg. CO_2

4.010 mg. substance gave 0.42 cc. N_2 (26.5° and 738 mm.)

$C_{21}H_{44}N_2O$ (353). Calculated. C 71.39,   H 12.18,   N 11.89
                         Found.   " 71.32, 71.54, " 12.11, 12.02, " 11.59

Isolation of a Second Alcohol from the Neutral Substance

Considerable difficulty was encountered in the isolation of other alcohols from the more soluble portion of the neutral substance. Direct crystallization failed to yield any pure components. The acetyl derivative was tried, but it had a low melting point and did not crystallize, the benzoyl derivative was an oily liquid at room temperature, and the phenylurethane could not be obtained in definitely crystalline form. Fractional distillation in a vacuum of the mixture of crude alcohols finally yielded 0.6 gm. of an alcohol which, however, was difficult to purify on account of its great solubility in organic solvents. It separated as fine colorless needles from methyl alcohol on cooling but generally indistinct crystalline burr-shaped aggregates were obtained. After several crystallizations from methyl alcohol the substance melted at 50°.

Rotation—0.3988 gm. of substance dissolved in ether and diluted to 10 cc. gave in a 1 dm. tube an average reading of +0.25°. Hence $[\alpha]_D^{19} = +6.27°$.

Oxidation of the Alcohol—The substance described above was oxidized in glacial acetic acid with chromic acid in the hope that the ketone would have better properties than the alcohol; 0.4 gm. of the alcohol yielded 0.35 gm. of a ketone which crystallized from methyl alcohol in beautiful large colorless plates. After two crystallizations the ketone melted at 48.49°, but further recrystallizations raised the melting point to 50–51°.

$C_{18}H_{36}O$ (268). Calculated. C 80.59, H 13.43
                    Found.   " 79.97, " 13.39

Semicarbazone—The semicarbazone was prepared and recrystallized from alcohol. The substance separated as colorless, thin, elongated plates which melted at 127–128°.

$C_{18}H_{38}N_3O$ (325).   Calculated.   C 70.15, H 12.00, N 12.92
Found.   " 70.45, " 12.32, " 12.29

The analytical values found for the ketone and semicarbazone agree approximately with the calculated composition of 2-octadecanone and its semicarbazone.   While the identification of the lower alcohol contained in leprosin is not absolutely established, the results obtained strongly suggest that it is d-octadecanol-2. The crystal forms of the ketone and of the semicarbazone were identical with those of 2-octadecanone and its semicarbazone previously prepared from d-octadecanol-2 of the timothy bacillus wax (2).   Mixed melting points of our alcohol, ketone, and semicarbazone with the corresponding compounds derived from the timothy bacillus wax showed a depression of about 1°.   It is possible that the lower alcohol isolated in this investigation contained a small amount of d-eicosanol-2; hence the ketone would contain some 2-eicosanone.

Examination of the Lead Salts—The lead salts, obtained as mentioned above, were first extracted with ether in order to remove any neutral substances, but no ether-soluble material was present because the ether extract on evaporation to dryness left practically no residue.   After this treatment the lead salts were shaken in ethereal suspension with water containing a little dilute nitric acid until everything dissolved.   The ethereal extract was washed with water until the washings were neutral, after which it was dried over sodium sulfate, filtered, and the ether distilled off.   The residue was a nearly colorless oil when warm and a white, hard wax-like mass at room temperature.   The material was readily soluble in warm ether, chloroform, benzene, and ethyl acetate and in boiling acetone, but very slightly soluble in ethyl or methyl alcohol or in cold acetone.   It separated from all solvents in the form of fine snow-white globular particles which showed no crystalline structure.

The substance was a mixture consisting mainly of a new hydroxy acid which will be designated by the name leprosinic acid and a small amount of higher ordinary fatty acids.   The latter were removed by repeatedly precipitating the substance from ether-acetone solution, from acetone, and by treatment with slightly warm alcohol, followed by further precipitations from acetone.   The product which remained after these treatments

represents crude leprosinic acid and amounted to about 20 per cent of the leprosin.

The material contained in the mother liquors was recovered and the higher fatty acids were separated as described below.

Examination of Higher Fatty Acids Recovered from Mother Liquors in Purification of Leprosinic Acid

The material recovered from the mother liquors in the purification of the leprosinic acid melted at 74–75° and after the melted substance had cooled it showed a crystalline structure. Attempts to purify the material by crystallization did not lead to any homogeneous products. Evidently several acids were present in the mixture.

A portion of the acids weighing 7.5 gm. was converted to the methyl ester and the latter was fractionated in a high vacuum, with a modified Widmer column. Three principal fractions were obtained, all of which were crystalline solids. The residue in the distilling flask, which probably consisted of the methyl ester of leprosinic acid, was not examined.

Fraction 1 weighed 0.7 gm. It melted at 39–40° and probably consisted largely of methyl stearate.

Fraction 2 weighed 1.9 gm. It melted at 50° but after repeated recrystallization from acetone the constant melting point of 56° was obtained. The purified ester was saponified and the free acid crystallized from acetone, from which solvent it separated in large colorless plates. After one crystallization the acid melted at 77–78° and this melting point could not be changed by numerous recrystallizations from acetone or ether.

Analysis—Found, C 78.00, H 13.07; mol. wt. by titration 356, 362

The values found are similar to those recorded previously for Fraction 4 of the fatty acids. Although the melting point remained constant after many recrystallizations, the acid is probably a slightly impure specimen of tetracosanic acid, although the analytical values agree with the calculated composition of a tricosanic acid.

Fraction 3—0.5 gm. of the ester was obtained. It melted at 60–61°; the melting point was not changed on recrystallization from acetone. The ester was saponified and the free acid was

recrystallized repeatedly from acetone and from a mixture of benzene and methyl alcohol until a constant melting point of 85–86° was attained. The acid crystallized in large colorless plates.

C$_{24}$H$_{48}$O$_2$ (368).   Calculated.   C 78.26, H 13.04, mol. wt. 368
                              Found.   " 78.03, " 13.15, "   " 372

The values found are in agreement with the calculated composition of a tetracosanic acid.

Leprosinic Acid—The crude leprosinic acid melted at 63–64°, [α]$_D$ in CHCl$_3$ = +4°. On analysis the following values were found: C 80.17, H 13.32; mol. wt. by titration 662, 664. Much time was spent in attempting to purify the acid but no definitely pure substance could be prepared. The evidence obtained indicates that we are dealing with one or more hydroxy acids of high molecular weight. However, the values found for the molecular weight by titration showed variations from about 660 to 1350 and no constant values could be obtained. It is possible that the differences in molecular weight depend upon lactone formation.

The properties of the derivatives that were prepared are briefly as follows: Acetyl derivative, m.p. 42–43°, [α]$_D$ in CHCl$_3$ = +4.6°; acetic acid on saponification from 6.1 to 6.9 per cent; methyl ester, m.p. 51–52°, [α]$_D$ in CHCl$_3$ = +4.1°. The free acid recovered from these derivatives melted at 62–63°, [α]$_D$ in CHCl$_3$ = +4.0°. The acid as well as its derivatives separated as fine, colorless, globular particles. Analyses of different fractions of the purified acid gave the following values: C 80.35, 80.98, H 13.21, 13.42.

When tested by the Hanus solution, the acid gave an iodine number of 6. In chloroform solution the acid slowly absorbs bromine and some hydrobromic acid is liberated. After a solution of the acid had stood for 6 days with an excess of bromine, a bromo derivative was isolated which melted at 54–55° and contained 17.6 per cent of bromine.

Further description of leprosinic acid will be deferred to a future time when more experimental work has been done.

The biological reactions of leprosin, leprosinic acid, and the higher secondary alcohols have been studied by Dr. F. R. Sabin and collaborators and will be reported in a separate publication. Dr. Sabin found that leprosinic acid was acid-fast.

SUMMARY

Leprosin, a neutral wax-like substance isolated from the leprosy bacillus, has been analyzed and found to consist of a complex mixture of solid glycerides and waxes.

The fatty acids liberated on saponification were myristic, palmitic, stearic, tetracosanic acids and a new hydroxy acid called leprosinic acid.

The neutral portion of leprosin after saponification consisted of both water-soluble and ether-soluble components. Glycerol was the only water-soluble substance that could be detected.

The ether-soluble unsaponifiable matter consisted of two higher, secondary, optically active alcohols. The less soluble alcohol was identified as d-eicosanol-2. A second alcohol, probably d-octadecanol-2, was also isolated.

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HELIUM SOLUBILITY IN BLOOD AT INCREASED PRESSURES

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Sayers, Yant, and Hildebrand (1925), assuming the helium solubility coefficient for blood to be the same as for water, suggested that breathing oxygen-helium mixtures would be effective in the treatment of compressed air illness. They stated that, because the solubility of helium is less than that of nitrogen, and that it diffuses more rapidly, the elimination of inert gases from blood and tissues would be more rapid if helium was substituted for the nitrogen in ordinary air.

Van Slyke, Sendroy, Hastings, and Neill (1928), Van Slyke and Sendroy (1928), and Van Slyke, Dillon, and Margaria (1934) have found that the assumption that different gases have the same relative solubility is incorrect for carbon dioxide, hydrogen, and nitrogen. Thus it seemed important to determine the solubility coefficient for helium in blood at atmospheric and increased pressures.

EXPERIMENTAL

The helium used for saturations was found by analysis to contain no other gases.

The ox or dog blood was oxalated and used immediately or chilled to nearly 0° and kept in tightly stoppered containers in the ice box until samples were saturated the following morning. Sufficient oxalate was added to make a 0.02 per cent solution.

Equilibrations under increased pressures were conducted in a recompression chamber described by Hawkins, Shilling, and Hansen (1935) with a mercury manometer connected to it so that pressures could be read accurately.

The solubility of helium in water at 1, 2, 4, and 6 atmospheres was determined to control the accuracy of the method used.

Method of Saturation

Saturation of the liquids with helium gas was carried out in a double tonometer according to the first saturation method of Austin *et al.* (1922). A 15 cc. tonometer was connected with a 1000 cc. tonometer by means of a short, large bore, rubber tube to make a double tonometer. The smaller vessel was filled with the liquid to be saturated, while the larger vessel was completely exhausted, washed out, and filled with helium gas. The liquid was then run into the larger tonometer and both tonometers were then rotated horizontally in the constant temperature water bath maintained at $38° \pm 0.05°$. The desired pressure was obtained by opening, at intervals, under water, the capillary stop-cock of the larger vessel, thus permitting the pressure of the gas, raised by warming in the bath, to come to the various pressures at which the equilibrations were being made. The gas was not renewed because of the limited time the operators could stay at the increased pressures. The total time allowed for saturation of the liquid was 30 minutes. Equilibration was apparently attained in this time.

After saturation the separation of the liquid phase in the small tonometer and the gas phase in the large tonometer was carried out as described by Austin *et al.* (1922). Both the gas and the liquid phase were analyzed within 1 hour after saturation by the following methods.

The carbon dioxide and oxygen tensions were determined by Haldane's method for gases, the remaining inert gas being measured as helium. At increased pressures, the larger tonometer was attached to a mercury leveling bulb and some of the gas forced out to allow for expansion of the remaining gas when the pressure was reduced to atmospheric.

The helium tension in the gas phase was calculated from the helium constant by the equation

$$P = \frac{\text{per cent helium in gas phase}}{100} \times (\text{manometer} - W)$$

where manometer is the corrected manometer pressure and W is the vapor tension of water at $38°$.

The total helium content of the liquids was determined by means of the manometric apparatus of (Van Slyke and Neill, 1924), the helium content being calculated by the equation

Volume per cent helium = $P \times$ helium factor (nitrogen factor)

At atmospheric pressure 5 cc. samples were taken for analysis when water or blood was equilibrated. Pressure readings for the measurement of the extracted helium gas were made after bringing the gas to a 0.5 cc. volume. Carbon dioxide and oxygen were absorbed by Fieser's air-free hyposulfite solution (1924) and the remaining gas measured as helium. Duplicate analyses were made on both liquid and gas phases of the equilibrated blood or water.

At the increased pressures, where equilibration was made, the blood or water to be analyzed was transferred from the tonometer to a special 2 cc. pipette with a 3-way stop-cock similar to that

TABLE I

Helium Solubility in Water at 38°

Experiment No.	He tension at 38°	He content	α
	mm.	*vol. per cent*	*cc. He per cc. solution*
1	723	0.80	0.0084
2	728	0.81	0.0085
3	1495	1.68	0.0085
4	3034	3.41	0.0085
5	3025	3.41	0.0085
6	4588	5.30	0.0087
7	4620	5.20	0.0085
8	4649	5.29	0.0086
Average...			0.0085

described by Van Slyke and Neill (1924). It was necessary to use this type of pipette to prevent any loss of helium as it escaped from the supersaturated solutions while the pressures were being reduced to normal. The pipette had a small bulb between the calibrated bulb and the lower stop-cock to prevent the liquid, on expansion of gas, from being driven down into the rubber tubing connecting the pipette to the mercury leveling bulb.

After return to atmospheric pressure the total gas and liquid contents were delivered quantitatively to the manometric apparatus. Oxygen capacity determinations were made on blood to determine hemoglobin content (Van Slyke and Neill, 1924). The water content of the blood was determined by drying 2 cc. samples of blood to constant weight at 110°. The solubility

coefficients α and α_0 were calculated as described by Van Slyke, Sendroy, Hastings, and Neill (1928).

TABLE II

Helium Solubility in Blood at 38°

Sample No.	O₂ capacity	H₂O content	He tension at 38°	He content of blood	α	α_0
	vol. per cent	gm. per cc.	mm.	vol. per cent	cc. He per cc. solution	cc. He per gm. H₂O
D-1	20.06	0.8965	699	0.83	0.0089	0.0099
			1466	1.79	0.0092	0.0103
			3028	3.54	0.0088	0.0098
			4556	5.59	0.0093	0.0104
D-2	20.14	0.8069	722	0.87	0.0090	0.0110
			1473	1.63	0.0084	0.0104
			4559	4.97	0.0083	0.0102
D-3	21.55	0.7767	714	0.84	0.0089	0.0115
			1462	1.74	0.0090	0.0116
			4434	5.07	0.0087	0.0116
D-4			706	0.79	0.0085	
			1534	1.80	0.0088	
			2937	3.26	0.0084	
			4553	5.16	0.0086	
O-1	18.18	0.8113	710	0.82	0.0087	0.0107
			1528	1.94	0.0096	0.0118
			2985	3.37	0.0085	0.0104
			4209	5.29	0.0095	0.0117
O-2	15.70	0.8223	1631	1.86	0.0086	0.0105
			3096	3.26	0.0080	0.0097
O-3	18.50	0.8079	704	0.83	0.0089	0.0110
			2990	3.38	0.0085	0.0105
			4850	5.82	0.0091	0.0113
O-4	20.01	0.8799	706	0.87	0.0093	0.0106
			3107	3.62	0.0088	0.0100
			4580	4.79	0.0079	0.0089
O-5	18.86	0.8122	703	0.83	0.0089	0.0116
			4523	5.10	0.0085	0.0105

Results

The results are presented in Tables I and II. It is seen from Table I that the solubility coefficient for helium in water at 38° is 0.085. Water saturated at 38° with helium pressures varying from 1 to 6 atmospheres (absolute) was found to contain the amounts of helium which would be calculated from Henry's law.

$$\text{Volume per cent dissolved helium} \quad \frac{100 \times P}{760}$$

The limits of error of the technique used are shown in Table I by the helium volume per cent determinations in duplicate saturations. The greatest deviation between the duplicate saturations was 0.03 volume per cent. The maximum deviation from the average helium solubility was 0.02 volume per cent.

Table II shows the helium solubility coefficient and the amounts of helium in volumes per cent dissolved in ox and dog blood equilibrated with helium pressures from 1 to 6 atmospheres. The helium solubility coefficient of the same blood equilibrated at pressures from 1 to 6 atmospheres (absolute) was found to be the same within the limits of experimental error.

The amount of helium dissolved in blood under helium pressures at 1, 2, 4, and 6 atmospheres (absolute) has been found directly proportional to the helium pressure. This agrees with the findings reported by Hawkins and Shilling (1936) for nitrogen solubilities in blood at increased pressures.

SUMMARY

Solubility coefficients of helium in whole blood of normal dogs equilibrated at atmospheric pressure were found to vary from 0.083 to 0.089, and in ox bloods from 0.080 to 0.091.

The amount of helium dissolved by whole blood under helium pressures varying from 1 to 6 atmospheres (absolute) has been found directly proportional to the helium pressure according to Henry's law.

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THE RECOVERY OF HYDROCYANIC ACID FROM FUMIGATED CITRUS LEAVES[*]

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The effect of hydrocyanic acid on living tissue is a current problem which is of special interest in southern California because of the varied results which are often obtained when fumigating citrus trees for the control of scale insects. Sometimes fumigation will result in injury to the foliage or the fruit, while, on the other hand, under apparently comparable conditions and with a lapse of only a day or two of time, no injury will be noticeable.

In order to determine, if possible, some of the factors which may be responsible for these results it was decided to measure by recovery the amount of HCN that citrus leaves of different ages will absorb under different conditions. Citrus tissues do not contain a cyanophoric glucoside. It was while attempting to recover HCN from the fumigated leaves that results of special interest were obtained. Mature leaves of *Citrus sinensis*, Osbeck were used in these tests.

Methods

Determination of HCN—The Liebig silver nitrate volumetric method was used for determining HCN. The addition of potassium iodide and ammonia to the solution just before titration began, as suggested by Denigès (3) and elaborated by Sharwood (5), was helpful in establishing a definite end-point. 0.1 gm. of KI and 0.15 to 3 ml. of NH_4OH in each 150 ml. aliquot produced good results. The use of larger amounts of NH_4OH in the presence of comparatively low concentrations of HCN tended to dissolve AgI and thus gave an erroneous end-point.

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The quantities of HCN dealt with in these tests were so small that titrating into a flask or beaker, or even using a nephelometric apparatus in a dark-room, proved to be entirely unsatisfactory. In order to overcome the difficulties encountered in the use of such methods, a Weston photronic photoelectric turbidimeter was devised and constructed (2). This apparatus has proved to be very satisfactory, not only as a turbidimeter in this work but as a colorimeter in other work. The presence of color or even turbidity in the solutions before the tests were made did not give trouble. The immersion of the leaves in an alkaline solution to recover the HCN resulted in the absorption of some leaf color and made the solution turbid from the dust on the leaves. The turbidity could not be entirely avoided by filtering.

Differences as small as 0.00054 mg. of HCN or 1 part in 200,000,000 could be readily determined. In detecting such small quantities 0.001 N AgNO$_3$ was used. However, for the regular tests 0.01 N AgNO$_3$ was used and no attempt was made to measure the recovered HCN to any such degree of accuracy.

Recovery of HCN—Trial tests were made to recover the HCN from fumigated leaves by a vacuum and by drawing air over them, but both methods were unsatisfactory. The next attempt consisted of submerging the leaves in a solution of 0.1 N NaOH. While in this solution they were shaken for $\frac{1}{2}$ hour on a mechanical shaker. By this method the expected amount of HNC was not recovered, so the more laborious method of placing the leaves in a dilute, non-volatile acid and catching the distillate in an alkaline solution was tried. This is the method that is generally recommended for the recovery of HCN from tissues. The leaves used in the tests were not comminuted but were used whole, just as they came from the trees. The tip of the condenser was extended and arranged in such a manner that it dipped into the alkaline solution in the receiver flask. A 2 per cent solution of tartaric acid was ordinarily used in the distillation flask. Water was constantly added to the distillation flask at approximately the same rate at which distillation took place, in order to keep the acid solution at its original concentration. Liquid hydrocyanic acid of known purity and concentration was used for fumigation and other tests.

As has been stated, the expected amount of HCN was not recovered when the leaves were submerged in an alkaline solution and shaken for 30 minutes. This was confirmed by placing 200 gm. of leaves in a 21 liter glass container, introducing a known amount of liquid HCN, waiting 45 minutes (the regular fumigation period), and then introducing an alkaline solution to absorb the HCN. The combined amount of HCN recovered from the container and from the leaves was only about one-half that recovered from the control container which contained no leaves. 95 per cent, or more, of the HCN was recovered from the control container.

It was because of the results mentioned in the preceding paragraph that the distillation method was tried. 200 gm. of fumigated leaves were distilled in 2 per cent tartaric acid and the distillate caught in 0.1 N NaOH. When the distillate was tested, no HCN could be detected. The method of procedure was then varied as follows: (1) Fumigated leaves were distilled in water instead of weak acid. (2) Unfumigated leaves were distilled in acid solution to which a known amount of HCN had been added. (3) Unfumigated leaves were distilled either in water or in acid solution, and the HCN put into the alkaline solution in the receiver flask before distillation began. (4) Instead of submerging the leaves in water or in acid solution, steam was passed over them and the distillate caught in a receiver flask containing HCN.

Not a trace of HCN could be detected in the distillate by any of these methods. The amount of HCN introduced into the distillation or receiver flasks was approximately the same as such a quantity of leaves would have access to in the usual fumigation practice.

From the results of the preceding tests it was very evident that the HCN was combining with some substance or substances carried over in the leaf distillate. In order to determine how much HCN might be combined in this manner 1 liter of distillate was obtained from 200 gm. of leaves submerged in 2 liters of a 2 per cent tartaric acid solution. The leaf substances contained in the 1 liter of distillate combined with 1.00 gm. (approximately 1.43 ml.) of liquid HCN in such a manner that it could not react

with AgNO₃ to form AgCN. To express it in another manner, this amount of HCN is approximately 65 times as much as could be recovered from a like quantity of leaves, fumigated under standard conditions, by submerging and shaking them in 0.1 N NaOH. It was thought that aldehydes might be combining with the HCN to form nitriles. However, tests showed that only comparatively small quantities of acetaldehyde, formaldehyde, and furfuraldehyde were extracted from the leaves by the distillation method used. The quantities either separately or combined were far from adequate to account for the large amounts of HCN which were tied up. That sugars did not interfere was proved by the fact that HCN could be fully recovered in the distillate when it had been added to a 2 per cent tartaric acid solution containing the finely divided tissues of mature, whole orange fruits. Full recovery of HCN was also obtained when distilled in the presence of cold-pressed or distilled orange oil and of pure citral; hence, citral could not have been responsible for the nonrecovery from the leaves.

DISCUSSION

In the past it has been pointed out that a number of factors influence the accuracy of the determination of the amounts of HCN that may be experimentally obtained from plants which contain cyanophoric glucosides. The work done in this field prior to 1916 was reviewed by Alsberg and Black (1) and it will not be reported or discussed here. These workers and Viehoever, Johns, and Alsberg (6) appear to have been the first to obtain further evidence on the problem by attempting to recover added HCN from macerated and non-macerated tissues. In working with such plants as *Prunus virginiana* and *Andropogon sorghum*, which contain cyanophoric glucosides, Alsberg and Black (1) found that if the finely divided tissues were distilled at once all of the added and autogenous HCN could be accounted for. If, on the other hand, the tissues were macerated, a certain amount of the HCN had disappeared, the amount being roughly proportional to the length of the maceration period. However, in testing the tissues of *Sambucus canadensis*, which do not contain a cyanophoric glucoside, it was found that all of the added HCN could be recovered not only by testing at once, but after allowing the tissues to be macerated for as long as 36 hours.

The results obtained with *Sambucus canadensis*, which does not contain a cyanophoric glucoside, are of interest when compared with *Citrus sinensis*, which also does not contain a cyanophoric glucoside. In the case of the latter, added HCN could not be recovered from the distillate obtained under any one of several conditions, until an excessive amount of it had been added to the distillate at the time of making the titration. Krieble and Peiker (4) have found that if too high a concentration of acid is used in the distillation flask, there is danger of hydrolyzing the HCN. It does not appear probable, however, that the 2 per cent tartaric acid, such as was used in most of these tests, could have hydrolyzed the HCN which had been absorbed by the citrus leaves during fumigation. The same results were obtained where no acid at all was used. Because of leaf acids the distillate partially neutralized the alkali in the receiver flask but not sufficiently to have allowed any HCN that might have come over to escape.

Although fumigated citrus leaves were able to absorb and retain HCN, so that much of it could not be recovered by submerging them in an alkaline solution, it was found that unfumigated leaves submerged in an alkaline solution containing HCN could not absorb any HCN from the solution.

The results obtained in this work indicate that the distillate from fresh citrus leaves contains comparatively large amounts of some substance or substances which are able to combine with or to break down HCN in such a manner that it cannot be detected. Whether they exist as such in a free state to so marked an extent in the living citrus leaf is not definitely known. However, considerable evidence is already at hand which indicates their presence in comparatively large quantities. Further results in this line will be presented in a later paper.

SUMMARY

A Weston photronic photoelectric turbidimeter, specially devised and constructed for the purpose, proved to be very satisfactory for determining hydrocyanic acid in solutions.

Evidence is presented which indicates that at least a portion of the HCN which enters the living citrus leaves during fumigation is quite rapidly and permanently fixed.

Hydrocyanic acid could not be recovered from fumigated citrus leaves by the acid distillation method because it combined with

some substance or substances in the distillate. Much more HCN than the leaves would be expected to absorb during the fumigation period had to be added before any could be detected in the distillate.

The evidence is not yet complete, but it does not appear that the aldehydes, sugars, or citral were entirely responsible for the disappearance of such large quantities of HCN.

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A STUDY OF THE ESTIMATION OF SODIUM IN BLOOD SERUM

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Without doubt the most satisfactory procedure for the quantitative determination of small amounts of sodium is that involving its precipitation as uranyl zinc sodium acetate, $(UO_2)_3ZnNa(CH_3COO)_9 \cdot 6H_2O$, first introduced by Barber and Kolthoff (2). Applications of this method to biological material have been numerous, since the triple salt is readily estimated by a variety of gravimetric, colorimetric, or volumetric procedures. Of these procedures that which appears to offer the greatest accuracy with the greatest sensitivity is the volumetric one employed by Kolthoff and Lingane (8). This, in brief, involves the reduction of hexavalent uranium in the triple salt to U^{IV} and some U^{III}; the latter is easily oxidized by air at room temperature to the quadrivalent state which is less susceptible to air oxidation. The quadrivalent uranium is then oxidized quantitatively to the hexavalent state with dichromate.

The application of this volumetric procedure to biological materials has not received the attention that the method merits. We therefore present, in the following pages, a method for the determination of sodium in blood serum based on these principles. Accurate results are obtainable on 0.2 ml. samples of serum. In proof we present parallel determinations on 10 to 20 ml. samples by the classical chloroplatinate method. For comparison we also present results obtained by the gravimetric method for estimating the uranium triple salt as described by Butler and Tuthill (3) and the older pyroantimonate method of Kramer and Gittleman (9). A comparison is also made of the values obtained on trichloroacetic acid filtrates of serum and ashed samples.

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Reagents—

Uranyl zinc acetate reagent, Barber and Kolthoff (2).   Solution A: To 75 gm. of uranyl acetate (2H₂O) add 400 ml. of water and 13 ml. of glacial acetic acid.   Solution B: To 350 gm. of zinc acetate (3H₂O) add 400 ml. of water and 10 ml. of glacial acetic acid. Heat to dissolve and mix the two solutions while hot.   The resulting mixture should be saturated at room temperature with uranyl zinc sodium acetate, excess solid being present.   An interval of several days before use is advisable.   Shake and filter each time immediately before using.

Glacial acetic acid, Salit (13).   Saturate glacial acetic acid with the triple salt.   Shake and filter each time immediately before using.

Ferric sulfate, 5 per cent.   5 gm. in 100 ml. of 2 N H₂SO₄.   Add dilute KMnO₄ solution dropwise till a faint pink tinge appears in order to insure the absence of ferrous iron.   The excess permanganate is now destroyed by boiling the solution vigorously for a few minutes.

Barium diphenylaminesulfonate, 0.2 per cent.   An aqueous solution of the salt supplied by the Eastman Kodak Company.

Trichloroacetic acid, 30 per cent.   Dissolve 30 gm. of Na-free acid in water and dilute to a total volume of 100 ml.

Potassium dichromate, 0.1 N and 0.025 N.   Dissolve 4.901 gm. of pure salt in water and dilute to 1 liter to obtain an exactly 0.1 N solution.   Prepare the weaker solution by dilution.   Solutions used in the present work were prepared from salt twice recrystallized and dried to constant weight at 140°.

Amalgamated zinc.   Dissolve 2.5 gm. of mercury in 25 ml. of HNO₃(1:1) and add 100 ml. of water and 250 gm. of c.p. granulated zinc (20 to 30 mesh, low in iron).   Shake vigorously for several minutes.   Wash thoroughly by decantation with water and air-dry.

Phosphoric acid, c.p. 85 per cent.

Sulfuric acid, 4 N and 2 N.

Caprylic alcohol.

The various sodium chloride solutions employed were made from Kahlbaum's best grade salt (*Zur Analyse mit Garantieschein*) dried at 120°.

Jones Reductor—Attempts to reduce the hexavalent uranium

of the triple salt completely to the quadrivalent or lower state by the action of titanous salts or plain zinc in acid solutions were unsatisfactory. Employment of a Jones reductor gave highly satisfactory results and, since this apparatus is extremely simple in construction, we do not hesitate to introduce it into biological work.

Construction—In brief its construction is as follows: To one end of a glass tube, 32 cm. long and 1 cm. in internal diameter, attach a glass stop-cock with a narrow bore delivery tube of 16 cm. length. To the other end seal an 8 cm. length of tubing, 2.5 cm. in internal diameter; thus forming a cup of about 40 ml. capacity into which solutions to be reduced are easily poured. The apparatus is now ready to be filled. With a few glass beads as a foundation place a layer of glass wool in the closed end of the tube, and follow this with a layer of asbestos about 1 mm. thick. This asbestos layer must be thick enough to keep back the zinc dust, and thin enough to permit free passage of solutions. Amalgamated zinc prepared as described above is now poured into the tube to give a column 25 cm. in height. A thin layer of glass wool now completes the construction.

Operation—The delivery tube of the reductor is inserted into an Erlenmeyer flask closed by a 2-hole rubber stopper. Suction is applied to the flask by means of the remaining aperture. The reductor is then cleaned by drawing through it 300 ml. of 2 N sulfuric acid. 100 ml. portions of acid drawn through after this should show no reducing action towards permanganate. If the zinc contains impurities, *e.g.* iron, a positive test may be encountered. In this case a blank value must be obtained on a volume of solution similar to that employed in the determination and subsequent results corrected accordingly. The ordinary c.p. grades of zinc with low iron content used in this work gave no blank corrections. The solution to be reduced is now poured into the bell of the reductor and suction applied until the column of zinc is barely covered with residual solution. Successive portions of 2 N sulfuric acid are now drawn through to transfer quantitatively the reduced solution to the flask, each time leaving the zinc column barely covered with liquid. After changing the receiving flask the apparatus is immediately ready for the reduction of another solution. When it is not in use, *e.g.* overnight, the sulfuric acid is

removed from the reductor by drawing water through it and storing with sufficient water to keep the zinc column well covered.   Before use the water is replaced by drawing through the reductor 50 ml. of 2 N sulfuric acid.   The zinc column should be kept immersed at all times, the level of fluid never being allowed to fall below the top of the zinc column whether the apparatus is in use or stored. If the zinc is allowed to become dry, 200 to 300 ml. of 2 N H_2SO_4 should be run through the apparatus before using it again.   For further details concerning the Jones reductor the reader should consult standard texts, *e.g.* (6).

All volumetric apparatus employed were calibrated.   Pipettes used for measuring serum were intercalibrated by weighing the volume of serum delivered and the results for the different methods were corrected accordingly.

Procedure

Removal of Protein.   Ashing[1]—To 1 ml. of serum contained in a platinum or a silica crucible add 1 ml. of 4 N sulfuric acid.   Evaporate on a steam bath for about $\frac{1}{2}$ hour, when a black residue is left. Place in a cold muffle furnace and gradually raise the temperature to 600°; avoid higher temperatures.   Heat until a white ash results.   Employing an electric furnace, calibrated with a thermocouple, we have found it convenient to start the furnace at the end of the day and allow it to run overnight.   With the rheostat set for 600° the cold furnace has warmed up gradually enough to prevent spattering, and ashing is complete by morning.   For 0.2 ml. samples of serum the procedure is the same except 0.5 ml. of 4 N H_2SO_4 is used.

Precipitation—To 1 ml. of serum contained in a 15 ml. centrifuge tube add exactly 5 ml. of distilled water and a fraction of a drop of caprylic alcohol.   (The alcohol is added to prevent subsequent adherence of precipitated protein to the sides of the tube and as shown by control experiments has no effect *per se* on the results.) Mix thoroughly and add 1 ml. of 30 per cent trichloroacetic acid, drop by drop, with constant stirring.   Centrifuge down the precipitated protein and draw off the supernatant fluid, of which a

[1] Attempts to employ wet ashing with sulfuric and nitric acids resulted in low and erratic results when the sodium was subsequently determined by the volumetric uranium procedure.

5 ml. aliquot is taken for analysis. The procedure for 0.2 ml. of serum is the same, the total volume being kept at 7 ml. by the addition of more water, 1 ml. of trichloroacetic acid still being employed. In working with 0.2 ml. samples we have preferred to obtain these by adding 0.5 ml. of serum to 2 ml. of water and taking 1 ml. of the mixture.

Determination of Sodium. Volumetric, on 0.2 Ml. of Serum—If the serum is ashed, add to the residue in the crucible 1 drop of 2 N sulfuric acid and transfer with two 0.5 ml. portions of water to a 15 ml. Pyrex centrifuge tube. Tubes should be kept clean by soaking in cleaning solution and well rinsed before use. Rinse the crucible with small portions of the uranium reagent. Crucibles that pour poorly may be smeared below the lip with vaseline. If a filtrate is employed, evaporate 5 ml. portions to incipient dryness in a crucible in order to adjust volume relations and proceed as outlined above.[2]

To the solution in the centrifuge tube add uranyl zinc acetate reagent to give a total of 10 ml. pouring down into the tube so as to wet the entire inner surface. Stir vigorously with a motor stirrer (see under "Results") for 10 minutes. Centrifuge down the sodium salt and decant the supernatant fluid, rotating the tube so that the entire inner surface is wetted. Allow the tube to stand inverted over filter paper for 5 minutes. Wipe the mouth of the tube dry and pour down the sides of the tube 10 ml. of the glacial acetic acid reagent, stirring up the precipitate. Centrifuge, decant, and drain again as above. Dissolve the precipitate in 15 ml. of 2 N sulfuric acid and transfer to the Jones reductor. Draw the solution through the reductor as described earlier at a rate such that the operation will be complete in about 45 seconds. Flush the reductor by drawing through it successively four 20 ml. portions of 2 N sulfuric acid, at the rate of approximately 40 ml. per minute. Disconnect the receiving flask from the reductor and draw air through the solution for 5 minutes. Add in the order named 5 ml. of a 5 per cent ferric sulfate solution, 5 ml. of 85 per cent phosphoric acid,

[2] Evaporation of the filtrate in the centrifuge tube in which the sodium precipitation is carried out leads to low results. We attribute this to a "dirtying" of the walls of the tube during evaporation, which in some way interferes with the complete precipitation of the sodium. Clean tubes are essential to good results. This is an example of the attention that must be paid throughout to details, when employing small amounts of sodium.

and 5 drops of barium diphenylaminesulfonate indicator,[3] swirling the flask vigorously after each addition.   Titrate with 0.025 N potassium dichromate until a violet color which persists for at least 30 seconds is obtained.   Subtract 0.06 ml. from the final value for the indicator correction (14).

Calculations—Ashed sample: Mg. of Na/100 ml. = (ml. of 0.025 N $K_2Cr_2O_7$ − 0.06 ml.) × 0.0958 × (100/0.2).   For the filtrate, calculate as above and multiply by $\frac{2}{3}$.

Volumetric, on 1.0 Ml. of Serum—If an ashed sample is employed, add 1 drop of 2 N sulfuric acid to the contents of the crucible and transfer to a well cleaned 50 ml. conical bottom centrifuge tube, using a total of 5 ml. of water.   For filtrates a 5 ml. aliquot is used directly.   Add 45 ml. of the uranyl zinc acetate reagent and stir by hand for 2 minutes after the appearance of a precipitate.   Allow to stand for 15 minutes.   Separate the precipitate from the excess reagent by centrifuging, decanting, and washing with 25 ml. of the glacial acetic acid reagent, as described above for the smaller serum sample.   Dissolve the precipitate in 30 ml. of 2 N sulfuric acid and draw through the Jones reductor.   Flush with five 20 ml. portions of 2 N sulfuric acid.   Observe the precautions and rates of flow previously described.   Aerate the reduced solution for 5 minutes.   Add in order and with shaking 15 ml. of ferric sulfate reagent, 5 ml. of phosphoric acid, and 5 drops of indicator solution.   Titrate with 0.1 N dichromate.   No indicator correction is necessary with this larger amount of sodium.

Calculations. Ashed Sample—Mg. of Na/100 ml. = ml. of 0.1 N $K_2Cr_2O_7$ × 0.3833 × 100.   For the filtrate, calculate as above and multiply by $\frac{2}{3}$.

It is unnecessary to remove phosphate before determining sodium by this method.   Assuming the phosphate to be precipitated as $UO_2 \cdot HPO_4$, 100 ml. of serum containing a total of 10 mg. of P will bind only as much uranium as 2.5 mg. of Na.   This is an error of about 0.7 per cent for serum containing 350 mg. of Na per 100 ml.   Analysis of solutions of known Na content and containing as much as 14 mg. of P per 100 ml. gave theoretical results (see Table IV).   To check this point further we have tried

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[3] The ferric salt and phosphoric acid are added in order to catalyze the slow reaction between dichromate and the indicator; a sharper end-point results.   (See reference (8) for further details.)

adding iron as recommended by Hald (5) for the removal of phosphate. Low results were obtained. The insoluble iron precipitate, when dissolved in acid, yielded a positive test for sodium. The fault may be ours in using an excess of iron, which the author states introduces difficulties in the solution of the ash. This point was not further investigated by us.

For comparison we have employed three other methods. These require brief comment. We have taken the classical Lindo-Gladding chloroplatinate method as our bed-rock of accuracy. The procedure used was that outlined by the Association of Official Agricultural Chemists (11). All samples were ashed in a muffle furnace as described above, 0.5 ml. of 4 N H_2SO_4 being used to each ml. of serum. For the removal of calcium, magnesium, and phosphate we first followed the short cut suggested by Mackay and Butler in Peters and Van Slyke (12). This procedure in our hands failed to remove completely these interfering salts and we returned to the longer but more satisfactory stepwise precipitation of the official method employing also ammonium oxalate. In order to obtain sodium-free ammonium hydroxide for use in this method we chose to prepare this reagent by passing tank ammonia gas into distilled water contained in a paraffin-coated Pyrex flask. The pyroantimonate method employed was that of Kramer and Gittleman as modified by Kerr (7). Ashed samples of serum were employed. Lastly we have employed the gravimetric method of estimating the triple uranium salt as outlined by Butler and Tuthill (3). All samples were ashed in a muffle furnace. Difficulty encountered by other workers (5, 13) in maintaining the alcohol wash solution saturated with the triple salt was also our experience.

Results

We have first investigated the effect of the length of time allowed for the precipitate to settle after formation and the type of stirring during precipitation on the accuracy of the results. The majority of previous workers, following Barber and Kolthoff, have allowed 1 hour to elapse for complete precipitation. Results given in Table I show that 15 minutes are sufficient time for complete precipitation of 1.0 to 5.0 mg. of sodium. For 0.5 mg. of sodium low results were obtained regardless of the elapsed time. These results were

obtained by the volumetric procedure outlined above for serum, hand stirring for 2 minutes being employed throughout. If, however, the precipitation of this small amount of sodium is carried out by motor stirring instead of hand stirring, a marked

TABLE I

Analysis of Sodium Chloride Solutions by Volumetric Procedure

Na taken	15 min.*			60 min.*		
	0.1 N K₂Cr₂O₇	Na found	Difference	0.1 N K₂Cr₂O₇	Na found	Difference
mg.	*ml.*	*mg.*	*per cent*	*ml.*	*mg.*	*per cent*
0.500	1.275	0.489	−2.2	1.265	0.485	−3.0
	1.270	0.487	−2.6	1.275	0.489	−2.2
1.00	2.60	1.00	0.0	2.59	0.99	−1.0
	2.63	1.01	+1.0	2.57	0.99	−1.0
2.00	5.27	2.02	−1.0	5.23	2.01	+0.5
	5.23	2.01	+0.5	5.22	2.00	0.0
5.00	13.03	4.99	−0.2	13.02	4.99	−0.2
	13.01	4.99	−0.2	13.02	4.99	−0.2

* Time that sodium precipitate was allowed to settle after formation.

TABLE II

Effect of Stirring on Accuracy of Volumetric Procedure When Determining Small Quantities of Sodium

In each experiment 0.500 mg. of Na was the amount taken.

Motor stirring			Hand stirring		
0.025 N K₂Cr₂O₇	Na found	Difference	0.025 N K₂Cr₂O₇	Na found	Difference
ml.	*mg.*	*per cent*	*ml.*	*mg.*	*per cent*
5.25	0.497	−0.6	5.16	0.489	−2.2
5.23	0.495	−1.0	5.21	0.493	−1.4
5.31	0.503	+0.6	5.15	0.488	−2.4
5.30	0.502	+0.4	5.18	0.490	−2.0
5.25	0.497	−0.6			
5.30	0.502	+0.4			

improvement in accuracy is obtained. Results of such an experiment are given in Table II. It is, therefore, apparent that stirring is more important than prolonged standing and we recommend motor stirring for 10 minutes and no further standing when dealing

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with 0.5 mg. or smaller quantities of sodium. Temperature control appears to be of minor importance. We have worked at normal room temperature, taking care only to prevent gross fluctuations during precipitation and washing.

TABLE III

Sodium Content of One Sample of Dog Serum Analyzed by Various Methods

The results are expressed in mg. of Na per 100 ml. of serum.

Serum ash						Serum filtrate			
Macrochloro-platinate	Pyro-anti-monate	Uranium				Uranium, volumetric			
		Gravi-metric	Volumetric						
20 ml.	2 ml.	1 ml.	1 ml.	0.5 ml.*	0.2 ml.	1 ml.	0.5 ml.*	0.2 ml.	1 ml.†
347	348	351	351	347	354	358	354	355	365
349	350	351	348	344	350	358	356	352	363
		349	349	353	355	354	356	353	367
		350	349	352	356	358	354	351	369
		347			347	356			
		349			352	354			
		345			353	358			
		347			355	360			
						357			
						356			
						356			
						355			
						355			
						354			
Average..348	349	349	349	349	353	356	355	353	366

* Procedure run through as for 1 ml. samples of serum.
† Serum added to 5 per cent CCl₃COOH. See discussion of results.

In Table III are summarized the results obtained on one sample of dog serum analyzed by various methods. Ashed samples gave practically identical values when analyzed by the macrochloro-

platinate, pyroantimonate, gravimetric, or volumetric uranium procedures. The reproducibility of the uranium procedure is attested to by the number of determinations all agreeing to within

TABLE IV

Sodium Content of Various Samples of Sera

The results are expressed in mg. of Na per 100 ml. of serum.

	Serum ash				Serum filtrate	
	Macro-chloro-platinate	Pyro-anti-monate	Uranium		Uranium, volumetric	
			Gravimetric	Volumetric		
	10 ml.	2 ml.	1 ml.	0.2 ml.	1 ml.	0.2 ml.
Beef......................	339	339	335	336	345	341
	340	339	339	335	347	346
Cat.......................	367	356	360		371	
	368	354	365		372	
		357				
		346				
Normal Human 1..........			324	325		331
			327	327		328
" " 2.........		316*	316		331	
		324*	322		328	
" " 3.........			320		336	
			327		335	
" " 4.........		308*	307		313	
			306		313	
Human 5†................			284		298	
			288		296	
Artificial serum‡..........	350	350	348		350	
	350	350	349		350	

* 1 ml. samples.

† Patient had received glucose intravenously.

‡ A non-protein solution containing, per 100 ml., 350 mg. of Na, 21 mg. of K, 11 mg. of Ca, 3.5 mg. of Mg, 14 mg. of P. All manipulations used for removal of protein from serum were also applied to this solution.

±1 per cent. The results on filtrates are not as satisfactory. Depending on the mode of precipitation and the ratio of serum volume to total filtrate volume, results varied from 1 to 5 per cent higher than those found for ashed samples. For example the

analyses reported in the last column of Table III were obtained on a filtrate prepared by adding 1 ml. of serum to 6 ml. of 5 per cent trichloroacetic acid. The results are 5 per cent too high. The analyses reported under the other columns headed "Filtrate" were obtained on a filtrate prepared by diluting the serum sample (0.2 to 1.0 ml.) to 6 ml. with water and adding 1 ml. of 30 per cent trichloroacetic acid. The results obtained here are only 1 to 2 per cent too high, the best agreement with the ashed values being obtained on the 0.2 ml. samples, where the serum dilution at the time of precipitation is greatest. Filtrates prepared with various other strengths of trichloroacetic acid and modifications gave values intermediate to those described above. Best results are therefore obtained on a filtrate prepared with 30 per cent trichloroacetic acid, according to the technique described under "Procedure." This peculiar effect will be discussed later.

Table IV contains the results obtained on various samples of sera. The findings are similar to those reported for dog serum in Table III. The values given by filtrates again run from 1 to 3 per cent too high. The results obtained by the pyroantimonate method are in good agreement with those obtained by the uranium and macrochloroplatinate methods and show that this older method is reliable if applied to ashed samples. One of us (1) has previously shown in the analysis of pancreatic juice that the pyroantimonate method gives values in agreement with those obtained by the macrochloroplatinate procedure.

<center>DISCUSSION</center>

The results obtained indicate that sodium may be determined in blood serum by the uranium procedure with an error of less than 1 per cent, if ashed samples are employed. Ashing in a muffle furnace is the procedure of choice and in our hands has required less attention from the analyst than any other method including filtrates. There is little to choose between the volumetric and gravimetric uranium procedures when 1 ml. samples are available. In fact, owing to its greater simplicity we are inclined to recommend the gravimetric method for samples of this size, though we feel that the volumetric procedure will yield more reproducible values. Occasionally the gravimetric method has yielded duplicates which do not agree as well as we would like; this has never been the case

with the volumetric procedure.   For samples smaller than 1 ml.
we recommend the volumetric uranium procedure.

Filtrates may be employed, if the highest accuracy is not re-
quired.   Results will be from 1 to 3 per cent too high, if the tech-
nique outlined under "Procedure" is followed.   This positive error
has been so consistently encountered we feel that an empirical
correction may be applied to the results, though we do not whole
heartedly recommend such practices.   The average positive
error with use of filtrates from 1 ml. and 0.2 ml. samples has been
respectively 2.8 ± 1.0 per cent and 1.2 ± 0.5 per cent.   If a cor-
rection based on these figures is applied, the preparation of the
filtrate must be as directed here, the trichloroacetic acid must be
proved Na-free as described below, and the volumetric procedure
must be employed.

We have endeavored to eliminate this annoying discrepancy
between filtrates and ashed samples by a stepwise investigation
of all possible sources of error.   Tests for sodium in the trichloro-
acetic acid were negative.   A solution approximating serum in its
inorganic composition and containing known amounts of sodium
(see Table IV) was run through the trichloroacetic acid procedure
used to precipitate the proteins in serum.   Subsequent analysis
for sodium gave values identical with untreated samples and with
the theoretical.   Trichloroacetic acid was found to cause no
interference in the volumetric estimation of the uranium; known
uranium solutions with and without trichloroacetic acid gave
identical values.   It was thought possible that the filtrates might
contain serum organic substances not precipitated by trichloro-
acetic acid but precipitated as uranium salts along with the
sodium.   Filtrates were therefore ashed before analyzing for
sodium.   High results were still obtained.   Investigation of other
minor details proved as futile.   The only explanation we can
offer for this phenomenon is that of volume displacement; *i.e.*, the
precipitated protein occupies a definite volume which actually
diminishes the volume of the filtrate and so increases the concen-
tration of the solutes.   In support of this view are the facts first,
that the error diminishes as the serum volume diminishes in rela-
tion to the total filtrate volume, and second, that the mode of
precipitation which gives the smallest bulk of precipitated protein
yields results nearest to the true value.

Upon looking over the literature it was found that other workers

had encountered a similar experience when employing trichloro-acetic acid filtrates. Van Slyke, Hiller, and Berthelsen (15) found serum filtrates yielded values 4 per cent higher than ashed serum samples, when analyzed for total base, and explained the error as due to volume displacement. Grigaut and Boutroux (4) report a +3 per cent error in their sodium determinations on serum filtrates and Oberst (10) found even a greater error, when determining sodium on filtrates of red cells where the protein bulk is large. A similar explanation of their results is offered by these workers. We are not aware of any cases in which a volume displacement effect has been noted on other than trichloroacetic acid filtrates or in which sodium was not involved in the determination. A careful investigation of this aspect with regard to other serum constituents would be of interest though probably difficult to achieve, especially in the case of organic material. Chloride offers the best opportunity. Wilson and Ball (16) found tungstate filtrates of serum and whole blood to give chloride values in good agreement with ashed samples determined gravimetrically. However, compensating errors (*e.g.*, the possible binding of the negative chloride ion as well as tungstate by the precipitated protein) which were not investigated might account for the agreement in this case.

SUMMARY

There is described a volumetric procedure for the determination of sodium in as little as 0.2 ml. of serum. The method is based on the precipitation of sodium as uranyl zinc sodium acetate, the reduction of the uranium in the precipitate to the quadrivalent state, and its subsequent quantitative oxidation with dichromate to the hexavalent condition. Serum ash or filtrate may be employed, though results with the latter give values 1 to 3 per cent too high, an effect ascribed to a volume displacement by the precipitated protein. Results on ashed samples agreed within 1 per cent with those found by the macrochloroplatinate procedure. The gravimetric uranium and pyroantimonate methods were also employed for the sake of comparison and found to be entirely reliable.

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COMPARATIVE STUDIES IN THE SULFUR METABOLISM OF THE DOG AND PIG

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Hele (1) has found that in the dog, maintained on a low protein diet, ethereal sulfates may be formed at the expense of endogenous sulfates. He has further shown, in support of Baumann's theory (2), that in the dog, the endogenous sulfates which are utilized in the synthesis of ethereal sulfates can be in part replaced by dietary inorganic sulfates. White and Lewis (3) obtained similar results with p-bromophenol.

In contrast to the dog, the pig does not seem readily to yield ethereal sulfates in the urine from the administered phenol, iodophenol, or chlorophenol. Supplementing the diet of the pig with l-cystine or Na_2SO_4 does not augment the synthesis of ethereal sulfates (4, 5). Furthermore, no p-bromophenylmercapuric acid could be either isolated (presumably by Baumann-Schmitz's method (6)) or detected by studying the sulfur partitions of the urine of pigs which were fed bromobenzene (5). Accordingly, Coombs and Hele (5) postulated that the pig, in contrast to the dog, does not utilize exogenous sulfur readily for detoxication purposes in general and does not, apparently, synthesize mercapturic acids in particular.

Recent studies have shown that isobarbituric acid readily yields ethereal sulfates in the urine of adult (7) and growing dogs (8), humans (9), and rabbits (10). Experiments were undertaken with the object of ascertaining the metabolism of isobarbituric acid in the pig. It also seemed of interest to see whether or not the synthesis of isobarbituric acid sulfate in dogs and pigs conforms to the theory of Baumann (2). Inasmuch as the studies of Sherwin and coworkers on pigs (4) indicated probable synthesis of p-bromophenylmercapuric acid by the animal, the experiments

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of Coombs and Hele (5) with bromobenzene were repeated and the p-bromophenylmercapturic acid isolated from the urine of pigs. The demonstration of the synthesis of p-bromophenylmercapturic acid by the pig has led us to investigate the metabolism of naphthalene in the same animal, particularly because naphthalene has recently been shown to yield 1-α-naphthalenemercapturic acid in rabbits (11), dogs (12), and rats (13).

<div align="center">EXPERIMENTAL</div>

Three adult female dogs and two 2 month-old female pigs were used in the experiments. The animals were confined to individual metabolism cages. The dogs received a protein-free diet, used by us in previous experiments (14), of the following composition: cane-sugar 84.3, Squibb's vitavose 11.6, salt mixture (15) 1.4, bone ash 2.7, butter fat 7, lard 17 parts. The diet contained 0.16 per cent nitrogen and 0.024 per cent sulfur. The diet yielded 60 to 65 calories per kilo of body weight of the dog. The pigs were fed Cowgill's diet (16), of the following composition: casein[1] 43.7, cane-sugar 40.6, Squibb's vitavose 11.6, salt mixture (15) 1.4, bone ash 2.7, butter fat 7, lard 17 parts. The diet contained 4.84 per cent nitrogen and 0.25 per cent sulfur. In addition to the diet, the pigs received 5 cc. of cod liver oil and 500 cc. of water, mixed with the food, *per diem*. The dogs and the pigs were allowed water *ad libitum*. The dogs were catheterized every 24 hours at 9 a.m.; the pig urine was collected without catheterization every 24 hours at 9 a.m. The food was given to dogs once a day, immediately after catheterization; the pigs were fed twice daily, at 9 a.m. and 12 noon. Bromobenzene and naphthalene were fed to the pigs at 9 a.m., mixed with the food. The pigs ate the food readily in spite of the odor and unpleasant taste of the supplements.

The plan of the experiments was to feed the animals the diets for several days, until the output of various urinary constituents showed little or no variation from day to day; then to feed isobarbituric acid mixed with the food and to continue the collection and the analysis of the urine until the output of the same urinary constituents had returned to normal. After several feedings of

[1] Casein No. 453 purchased from The Casein Manufacturing Company of America, Inc.

isobarbituric acid to the animals, the diets were supplemented successively by *l*-cystine, cysteine hydrochloride (neutralized with Na₂CO₃), *dl*-methionine, and Na₂SO₄, and the animals were allowed to balance on the supplemented diets. The feedings of isobarbituric acid were then made as before.

The same analytical methods were employed as described in our previous reports (14). *p*-Bromophenylmercapturic and 1-*α*-naphthalenemercapturic acids were isolated from the urine of pigs by the method of McGuinn and Sherwin (17). *l*-Cystine was prepared from human hair, with the necessary precautions against racemization. Cysteine hydrochloride and *dl*-methionine were purchased from the Eastman Kodak Company. Bromobenzene was redistilled and naphthalene resublimed before use. Isobarbituric acid was synthesized according to the procedure of Davidson and Baudisch (18). The purity of all compounds was checked by analysis.

DISCUSSION

For the sake of economy of space, complete experiments with isobarbituric acid on one dog and one pig only are presented in Tables I and II respectively. Identical results were obtained with the other animals. The metabolic studies on pigs with bromobenzene and naphthalene are illustrated in Table III.

Experiments with Isobarbituric Acid on Dogs—The data in Table I seem to indicate that the dog readily yields ethereal sulfates from isobarbituric acid while maintained on a protein-free diet. On the day of feeding isobarbituric acid, the output of total sulfur greatly increased. The increase is almost completely accounted for by the rise in the output of ethereal sulfates. The rise in the output of total sulfur was accompanied by only a small rise in the output of total nitrogen, in no case, however, exceeding the amount of nitrogen fed as isobarbituric acid. The data in Table I seem to indicate that in the dog maintained on a protein-free diet, isobarbituric acid is not broken down to yield urea. The formation of ethereal sulfates, under these conditions, apparently takes place at the expense of endogenous sulfur. The removal of this endogenous sulfur from the body for detoxication purposes is not apparently accompanied by the elimination of nitrogen in the urine. The data obtained by White and Lewis (3) with *p*-bromophenol point to the same conclusion.

Supplementing the protein-free diet with *l*-cystine, cysteine, *dl*-methionine, or Na_2SO_4 and feeding the dogs maintained on these

TABLE I

Metabolism of Isobarbituric Acid in the Dog

The initial weight of the dog was 9.7 kilos; at the end of the experiments (after 47 days), 9.3 kilos.

Intake		Supplement	Urinary output				
Nitro-gen	Sulfur		*Total* N	*Urea* N	Inor-ganic sulfate S	Ethe-real sulfate S	*Neutral* S
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0.20	0.024	None	0.97	0.47	0.016	0.014	0.060
			1.00	0.49	0.012	0.010	0.060
			1.37	0.53	0.000	0.107	0.080*
			1.01	0.50	0.000	0.008	0.070
			0.93	0.44	0.003	0.010	0.067
0.20	0.249	Na_2SO_4, 1.0 gm.	1.54	0.89	0.281	0.018	0.057
			1.76	1.09	0.270	0.019	0.054
			2.09	1.12	0.145	0.207	0.046*
			1.82	1.04	0.245	0.029	0.060
			1.70	0.91	0.250	0.030	0.050
0.31	0.249	Cysteine HCl, 1.29 gm.	1.10	0.63	0.090	0.016	0.056
			1.23	0.67	0.151	0.018	0.056
			1.57	0.62	0.023	0.142	0.095*
			1.17	0.61	0.142	0.023	0.097
			1.39	0.63	0.170	0.017	0.124
0.31	0.249	*dl*-Methionine, 1.13 gm.	0.92	0.36	0.157	0.013	0.061
			0.90	0.33	0.198	0.019	0.063
			1.24	0.28	0.039	0.152	0.058*
			1.00	*0.36*	*0.162*	*0.022*	*0.064*
0.30	0.249	*l*-Cystine, 0.85 gm.	*0.96*	*0.37*	*0.171*	*0.019*	*0.070*
			1.00	0.43	0.084	0.017	0.071
			0.93	0.40	0.138	0.019	0.083
			1.33	0.44	0.024	0.153	0.100*
			1.00	0.40	0.176	0.027	0.097
			0.90	0.37	0.192	0.020	0.100

* 2.5 gm. of isobarbituric acid were fed with the food; 0.55 gm. of N.

diets isobarbituric acid resulted in a 50 to 100 per cent increase in the output of ethereal sulfates, as compared to the amounts of ethereal sulfates formed from isobarbituric acid when fed to dogs

maintained on the unsupplemented protein-free diet. It is apparent from the data of Table I that the supply of endogenous sulfur which is used for the synthesis of isobarbituric acid sulfate

TABLE II

Metabolism of Isobarbituric Acid in the Pig

The initial weight of the pig was 9 kilos; after 38 days, 10.0 kilos.

Intake			Urinary output				
Nitrogen	Sulfur	Supplement	Total N	Urea N	Inorganic sulfate S	Ethereal sulfate S	Neutral S
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
6.10	0.310	None	3.08	2.00	0.007	0.013	0.064
			2.96	1.87	0.002	0.011	0.059
			3.27	1.91	0.008	0.031	0.080*
			2.91	1.81	0.000	0.010	0.071
			2.90	1.80	0.001	0.011	0.076
6.10	0.535	Na₂SO₄, 1.0 gm.	3.16	1.97	0.236	0.011	0.055
			3.10	1.93	0.247	0.020	0.053
			3.79	1.90	0.214	0.049	0.067*
			3.20	1.76	0.229	0.021	0.060
			3.27	1.83	0.231	0.019	0.050
6.10	0.535	Cysteine HCl, 1.29 gm.	3.90	2.99	0.060	0.028	0.110
			3.86	2.90	0.164	0.023	0.140
			4.30	3.00	0.119	0.061	0.169*
			4.00	2.96	0.166	0.030	0.156
			3.73	2.60	0.160	0.020	0.141
6.10	0.535	*l*-Cystine, 0.85 gm.	3.92	2.90	0.086	0.021	0.113
			4.00	2.93	0.173	0.020	0.117
			4.41	3.06	0.127	0.056	0.143*
			3.96	2.83	0.177	0.027	0.126
			4.10	3.10	0.162	0.025	0.130
6.10	0.535	*dl*-Methionine, 1.13 gm.	3.92	2.97	0.118	0.021	0.100
			4.00	2.97	0.157	0.023	0.106
			4.33	3.09	0.113	0.059	0.138*
			4.16	3.00	0.170	0.028	0.120
			4.01	3.10	0.176	0.020	0.110

* 2.5 gm. of isobarbituric acid were fed with the food; 0.55 gm. of N.

can to a large extent be replaced by exogenous organic and inorganic sulfur. In this respect the metabolism of isobarbituric acid in dogs is similar to that of indole, and phenol and its derivatives (1, 3, 5).

Experiments with Isobarbituric Acid on Pigs—The data presented in Table II seem to indicate that the pig, unlike the dog, does not readily synthesize ethereal sulfates from isobarbituric acid, in spite of the fact that the diet furnished an ample supply of various sulfur-containing compounds. The increase in the output of ethereal sulfates on the day of feeding isobarbituric acid is much

TABLE III

Metabolism of Bromobenzene and Naphthalene in Pigs

	Intake		Urinary output						
	Nitrogen	Sulfur	Total N	Urea N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Neutral S
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Pig 1, ♀; 10 kilos	6.1	0.31	4.00	3.00	0.241	0.116	0.077	0.039	0.125
	6.1	0.31	3.82	2.70	0.253	0.123	0.090	0.033	0.130
	6.1	0.31	4.20	3.10	0.334	0.173	0.066	0.107	0.161*
	6.1	0.31	4.16	3.16	0.272	0.103	0.032	0.071	0.169
	6.1	0.31	3.63	2.49	0.280	0.150	0.111	0.039	0.130
	6.1	0.31	3.92	3.00	0.264	0.133	0.095	0.038	0.131
	6.1	0.31	4.02	3.00	0.250	0.129	0.097	0.032	0.121
	6.1	0.31	4.40	3.46	0.297	0.109	0.040	0.049	0.188†
	6.1	0.31	3.90	2.77	0.241	0.101	0.049	0.052	0.140
	6.1	0.31	3.79	2.76	0.270	0.135	0.102	0.033	0.135
Pig 2, ♀; 13.3 kilos	7.7	0.41	5.31	4.10	0.220	0.110	0.070	0.040	0.110
	7.7	0.41	5.20	4.00	0.233	0.115	0.083	0.032	0.118
	7.7	0.41	5.66	4.63	0.262	0.096	0.006	0.090	0.166*
	7.7	0.41	5.02	3.92	0.212	0.070	0.030	0.040	0.142
	7.7	0.41	5.12	3.90	0.247	0.124	0.097	0.027	0.123
	7.7	0.41	5.23	4.00	0.266	0.140	0.109	0.031	0.126
	7.7	0.41	5.29	4.16	0.250	0.130	0.102	0.028	0.120
	7.7	0.41	5.72	4.60	0.228	0.079	0.022	0.057	0.149†
	7.7	0.41	5.00	4.27	0.216	0.042	0.008	0.034	0.174
	7.7	0.41	5.00	4.10	0.276	0.156	0.136	0.020	0.120

* 2.0 gm. of bromobenzene were fed with the food.

† 2.0 gm. of naphthalene were fed with the food.

smaller than the one usually obtained on dogs or other animals studied so far (6–9), which were fed similar amounts of isobarbituric acid. The synthesis of the ethereal sulfates, however small, seems to take place in pigs also at the expense of inorganic sulfates.

Experiments with Bromobenzene and Naphthalene on Pigs—The

data shown in Table III seem to indicate that the metabolism of bromobenzene and naphthalene in pigs is similar, at least qualitatively, to that in dogs (3, 12) and rabbits (11, 19). On the day of feeding bromobenzene and naphthalene to pigs, a marked rise in the output of neutral sulfur and ethereal sulfates in the urine was observed. The rise in the output of neutral sulfur in the urine of pigs seems to be smaller than that observed in dogs (3, 12) which were fed similar doses of bromobenzene and naphthalene. Pending the application of the method for.the determination of mercapturic acids in dog urine (20) to pig urine, we prefer not to assume that the neutral sulfur rise is a measure of the extent of the synthesis of mercapturic acids in the pig.

p-Bromophenylmercapturic and 1-α-naphthalenemercapturic acids were isolated from the urine of each pig separately after feeding them bromobenzene and naphthalene respectively. The analysis of the substances gave the following results.

	p-Bromophenyl-mercapturic acid			1-α-Naphthalenemercapturic acid			
	M.p.	Nitrogen	Sulfur	M.p.	Nitrogen	Sulfur	$[\alpha]_D^{25}$
	°C.	per cent	per cent	°C.	per cent	per cent	degrees
Calculated........	152	4.40	10.06	170	4.84	11.09	
Found............	152	4.29	10.00	170	4.72	11.00	−25 (1% in absolute alcohol)

The yields of p-bromophenylmercapturic and 1-α-naphthalenemercapturic acids were rather small; namely, 35 and 41 mg. from each 2 gm. dose of bromobenzene and naphthalene, respectively. As in the case of rabbits (11), dogs (12), and rats (13), acidification of the urine of pigs fed naphthalene with one-tenth its volume of concentrated HCl yielded, after 2 or 3 hours standing at room temperature, free naphthalene. The naphthoresorcinol test for ·glucuronates in the.urine of pigs which were fed isobarbituric acid, bromobenzene, and naphthalene was intensely positive in all cases.

SUMMARY

1. Isobarbituric acid, when fed to dogs maintained on a protein-free diet, readily yields ethereal sulfates in the urine.

2. The administration of *l*-cystine, *dl*-methionine, cysteine, or Na₂SO₄, together with isobarbituric acid, to the same dogs, similarly maintained, results in a still further increase in the output of ethereal sulfates in the urine.

3. In contrast to the dog, the pig does not yield ethereal sulfates from isobarbituric acid readily, and various sulfur-containing compounds administered simultaneously with isobarbituric acid do not augment the synthesis of ethereal sulfates in the pig.

4. The pig, like the dog, rabbit, and rat, synthesizes *p*-bromophenylmercapturic and 1-α-naphthalenemercapturic acids from bromobenzene and naphthalene respectively.

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THE GUM FROM LEMON TREES*

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This paper, dealing with the composition of lemon gum which is
a pathological product, will be followed by other papers dealing
with normal polyuronide materials from lemon wood. The in-
vestigation bears on the general question of the composition,
location, and origin of the polyuronides of wood.

A polyuronide normally present in wood may originate from at
least three different sources. (*a*) It may be synthesized directly
from glucose or other sugar. (*b*) It may result from transforma-
tion of a true polysaccharide such as starch. (*c*) It may be formed
from some other polyuronide. A decision as to which of these
possibilities is the actual source of the polyuronide can only be
reached by consideration of all the evidence available. One line
of evidence lies in a study of plant gums. These are typical
pathological polyuronides. Many microorganisms, including
both bacteria and molds, synthesize polysaccharide materials
when grown on media containing simple sugars (1). Some bac-
teria are even known to synthesize polyuronide materials from
simple sugars (2). Fawcett (3) has discussed the nature and
cause of bark diseases of citrus trees. He concludes that gum
formation on these trees is due chiefly to diseases, especially of
the bark, and that these diseases are caused by microorganisms,
many of which are fungi. Careful studies of the composition of
lemon gum and of the fungi that lead to its formation are desirable

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because of the light they might throw on the origin of the polyuronides in general.

Occurrence and Physical Properties of Lemon Gum—Lemon gum exudes from lemon trees during gummosis (3). The dry gum is a brownish yellow translucent solid which can be ground to a powder. It has a faint odor but is almost tasteless. It dissolves in 3 parts of cold water to form a viscous solution. When very concentrated solutions of the gum are heated, they often form a semisolid gel. The gum does not reduce Fehling's solution until after it has been hydrolyzed by hot dilute acids. It consists of a uronic acid to which is attached an ether-linked methoxyl group. The aldehyde group of the uronic acid is attached by a glucosidic linkage to a series of molecules of *d*-galactose. The latter are attached by glucosidic linkages to a series of molecules of *l*-arabinose. The composition of the purified gum corresponds to 1 molecule of a methylated uronic acid plus 2 molecules of *d*-galactose plus 2 molecules of *l*-arabinose minus 5 molecules of water. Since, however, salts were obtained in which 1 molecule of a methylated uronic acid is combined respectively with 1, 2, 3, and 4 molecules of *d*-galactose, it appears that this simple formula must be doubled. This would indicate that the gum consists of 2 molecules of a methylated uronic acid plus 4 molecules of *d*-galactose plus 4 molecules of *l*-arabinose minus 10 molecules of water.

Analysis and Purification of Lemon Gum—Selected samples of the gum were dried and analyzed with the results shown in Table I. The ash seemed to consist almost wholly of calcium oxide and it is reported as such in Table I. The very slight solubility in alcohol of the precipitate of furfural phloroglucide indicates that methyl pentoses are absent.

To purify the gum it was dissolved in 10 parts of cold water and filtered. The filtrate was mixed with 4 times its volume of 95 per cent alcohol. The granular precipitate was filtered and washed with alcohol. On analysis the purified material gave the results shown in Table I. In this table the per cent arabinose has been corrected for the furfural phloroglucide given by the uronic acid. The theoretical results given in Table I are for the calcium salt of an acid composed of 2 molecules of a methylated

uronic acid plus 4 molecules of d-galactose plus 4 molecules of l-arabinose minus 10 molecules of water. The only serious discrepancy between the theoretical composition and the results obtained is the percentage of galactose. It is known that the mucic acid method for determining galactose gives low results, especially when a methylated uronic acid is joined directly to the galactose. Apparently under these conditions this molecule of galactose gives very little mucic acid but is converted largely into oxalic acid.

The free acid was prepared from lemon gum by adding an excess of hydrochloric acid to a filtered water solution of the gum. The organic acid was precipitated by addition of 4 volumes of 95 per cent alcohol. To free the material from traces of hydrochloric

TABLE I

Analysis of Lemon Gum

		CO_2	CaO	Galactose	Arabinose	Methoxyl	Rotation $[\alpha]_D^{20}$
		per cent	per cent	per cent	per cent	per cent	degrees
Crude gum	Found	5.6	4.02	34.2	35.6	2.9	
Pure "	"	5.51	3.88	38.6	35.0	3.80	+20.7
	Theory	5.52	3.51	45.16	37.64	3.89	
Free acid	Found	5.60	0.11	41.3	32.6	4.42	+20.6
	Theory	5.65	0.00	46.3	38.6	3.98	

acid it was dissolved in water and reprecipitated by alcohol a total of eight times. The granular powder was washed with alcohol and dried. It gave no test for chlorides. The results obtained on analysis are given in Table I. The indications are that during the preparation of the acid a slight amount of l-arabinose was split off the molecule. This is to be expected, since l-arabinose readily comes off on warming the acid solution to 80°.

Hydrolysis of Lemon Gum—The gum was mixed with 8 times its weight of 4 per cent sulfuric acid and allowed to stand with occasional shaking until it had dissolved. The solution was heated to 80° for 6 hours. It was then neutralized with excess of barium carbonate and filtered from insoluble material. The filtrate was concentrated in vacuo and the products separated by treatment with alcohol into the salt of a uronic acid-sugar com-

pound, which was insoluble in alcohol, and an alcohol solution of the sugars. A portion of the salts obtained by hydrolysis at 80° was dissolved in 6 times its weight of 4 per cent sulfuric acid and heated in a bath of boiling water for 20 hours. The resulting solution was separated as described above into an alcohol-insoluble salt and an alcohol solution of the sugars. A portion of the resulting salts was mixed with 6 times its weight of 4 per cent sulfuric acid and heated in an autoclave at 120° for 12 hours. This solution was also separated into an alcohol-insoluble salt and an alcohol solution of the sugars. The products of hydrolysis under different conditions were examined as described below. The duration of hydrolysis was varied in the course of the investigation. When the purified gum was hydrolyzed for 6 hours at 80° no insoluble material remained. This indicates that no insoluble X body is present in the gum.

The Sugars—The alcohol solutions of the sugars obtained under different conditions were concentrated, separately, and the sugars crystallized from glacial acetic acid. The sugar obtained by hydrolyzing the gum at 80° for 6 hours consisted chiefly of *l*-arabinose. This was identified by its melting point, 154°, and by its rotation, $[\alpha]_D^{25} = +104°$. It was further identified by conversion to the characteristic diphenylhydrazone, melting at 199°. Small amounts of crystalline *d*-galactose were obtained from this sugar solution. This was identified by its rotation, $[\alpha]_D^{25} = +79°$, and by oxidation to mucic acid which melted at 218°. When the gum was hydrolyzed at 80° for 18 hours instead of 6 hours, a relatively larger amount of *d*-galactose was present in the sugar mixture. The sugars obtained by hydrolysis of the salts in the bath of boiling water gave large amounts of crystalline *d*-galactose and small amounts of crystalline *l*-arabinose. The sugars obtained by final hydrolysis of the salts in the autoclave consisted chiefly of crystalline *d*-galactose. The presence of traces of *l*-arabinose in this syrup was established by the diphenylhydrazone. It is evident that *l*-arabinose is first hydrolyzed off the molecule, leaving *d*-galactose still attached. The last molecule of *d*-galactose is very difficult to split off from the methylated uronic acid. None of the syrups obtained at any time fermented with ordinary yeast in a short time. No other sugars could be detected.

The Uronic Acid—The presence of a uronic acid in lemon gum was established by the naphthoresorcinol test (4) and by the yield of carbon dioxide by the method of Lefèvre and Tollens (5). The presence of an ether-linked methoxyl group in the gum as well as in all the salts obtained by hydrolysis of the gum was established by the procedures of von Fellenberg (6) and Denigès (7). This methoxyl group must be attached to the uronic acid, since it is not found with the sugars but always remains with the salts, even after hydrolysis in the autoclave. The identification of the uronic acid is made difficult by the presence of the methoxyl group. Neither saccharic nor mucic acid could be obtained from any of the salts by treatment with bromine and hydrobromic acid according to the method of Heidelberger and Goebel (8). When the salts obtained by hydrolysis in the autoclave are oxidized with nitric acid, small amounts of mucic acid result. This is to be expected, since it is difficult to hydrolyze off the last molecule of *d*-galactose from the methylated uronic acid. In one case small amounts of potassium acid saccharate were apparently obtained by oxidation of salts that had been hydrolyzed in the autoclave. This would indicate the presence of *d*-glucuronic acid. This observation has not yet been confirmed.

Uronic Acid-Sugar Compounds—Since the uronic acid-sugar compounds obtained by hydrolysis of lemon gum do not crystallize, it is difficult to isolate pure compounds from the mixtures. However, by dissolving the salts in water and fractionally precipitating them with alcohol it is possible to obtain materials that approximate closely the composition of definite compounds. The process must be repeated several times until products of constant composition are obtained. In general the larger the molecule the less soluble it will be in the mixture of alcohol and water. Table II gives the results obtained on analysis of eight uronic acid-sugar compounds resulting from the hydrolysis of lemon gum. In Table II the letters correspond to the following compounds: Compound A. The barium salt of methoxyuronic acid plus 1 molecule of *d*-galactose. It was obtained by hydrolysis of the gum in a bath of boiling water for 12 hours. Compound B. The barium salt of the dibasic acid obtained by oxidizing Compound A with barium hypoiodite. Compound C. The barium salt of methoxyuronic acid plus 1 molecule of *d*-galactose plus

Gum from Lemon Trees

1 molecule of *l*-arabinose. Compound C and those that follow were obtained by hydrolysis of lemon gum at 80° for 9 hours. Compound D. The barium salt of methoxyuronic acid plus 2 molecules of *d*-galactose. Compound E. The barium salt of methoxyuronic acid plus 2 molecules of *d*-galactose plus 1 molecule of *l*-arabinose. Compound F. The barium salt of methoxyuronic acid plus 3 molecules of *d*-galactose. Compound G. The barium salt of methoxyuronic acid plus 4 molecules of *d*-galactose. Com-

TABLE II

Composition of Uronic Acid-Sugar Compounds from the Hydrolysis of Lemon Gum

Compound		CO_2	Barium	Galactose	Arabinose	Methoxyl	Rotation $[\alpha]_D^{28}$
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*
A	Found	9.8	15.42	24.0		6.50	+74.3
	Theory	10.03	15.7	41.0		7.07	
B	Found	7.75	26.20	19.0			
	Theory	8.44	26.36	34.5			
C	Found	7.81	11.55	17.0	32.3		+92
	Theory	7.73	12.06	31.6	26.3		
D	Found	7.50	11.65			4.90	+69.2
	Theory	7.34	11.45			5.17	
E	Found	6.35	10.3	40	13.3	4.90	+51.2
	Theory	6.01	9.36	49.1	20.5	4.24	
F	Found	5.40	8.81	54	7.74	4.95	+42.2
	Theory	5.77	9.02	71	0	4.07	
G	Found	4.9	8.63	65.5		3.67	+39.7
	Theory	4.76	7.44	78		3.36	
H	Found	5.04		76		4.36	+40.4
	Theory	5.14		84.1		3.61	

pound H. The free acid obtained by treating Compound G with sulfuric acid. In general the rotation of the above compounds increases with decrease in the number of *d*-galactose molecules that are attached to the uronic acid.

Structure of Lemon Gum—A few conclusions can be drawn relative to the structure of the gum. (*a*) The methoxyl group is ether-linked to the uronic acid and not ester-linked. (*b*) The aldehyde group of the uronic acid is joined by a glucosidic linkage to an alcohol group of *d*-galactose. This is evident from the fact

that aldobionic acid, Compound A in Table II, can be oxidized to dibasic acid, Compound B, and still contain a uronic acid. (*c*) A chain of 4 molecules of *d*-galactose is attached to the methoxy-uronic acid. (*d*) Little definite can be said yet as to the mode of union of the molecules of *l*-arabinose. However, 1 molecule of this sugar seems to be attached to the molecule of *d*-galactose that is attached to the uronic acid.

<div align="center">DISCUSSION</div>

It is difficult to understand how a substance with the composition of lemon gum could originate from either the pectic substances or the hemicelluloses that have been isolated from lemon wood (9). In order for a pectic substance to be converted to lemon gum it would seem necessary to reduce the *d*-galacturonic acid back to *d*-galactose. It is very doubtful whether this process occurs in the present case. The hemicelluloses obtained from lemon wood are composed very largely of *d*-xylose combined with a methoxyuronic acid. It appears very unlikely that such a body would be readily converted to lemon gum.

Fawcett (3) states that the gum is formed not from the wood portion of the tree but from the inner portion of the bark. The fungi attack chiefly the cambium region of citrus trees. This region contains both starch and simple sugars. By using a medium of corn-meal-agar Fawcett kept alive for 8 years the culture of a fungus that causes gummosis. This medium certainly contained large amounts of starch. The evidence available indicates that the fungi which are causing gummosis in this case are transforming either starch or some simple sugar, quite possibly *d*-glucose, into the gum. A study of the compounds formed by cultures of these fungi on carbohydrate-containing media should throw more light on the origin of the gum.

<div align="center">SUMMARY</div>

The composition of lemon gum corresponds to a compound of 1 molecule of a methylated uronic acid plus 2 molecules of *d*-galactose plus 2 molecules of *l*-arabinose minus 5 molecules of water. To account for some of the salts obtained on hydrolysis of the gum it seems necessary to assume that this simple formula is doubled. The methylated uronic acid is attached by a gluco-

sidic union from its aldehyde group to a series of molecules of
d-galactose. In no case was a molecule of *l*-arabinose found
attached directly to the uronic acid. *d*-Galactose, *l*-arabinose,
and a series of uronic acid-sugar compounds were obtained from
the products of hydrolysis of the gum. The evidence indicates
that lemon gum is not formed from pectic substances nor from
hemicelluloses in lemon wood but from some of the simple sugars
or polysaccharides present in the inner portion of the bark of
citrus trees.

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QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

II. APPARENT ACID DISSOCIATION CONSTANTS IN AQUEOUS FORMALDEHYDE SOLUTION*

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Acid dissociation constants in aqueous formaldehyde solution of only seven amino acids (1, 2) and no peptides have been previously reported. It seemed desirable, therefore, to determine the constants of some peptides and other amino acids by means of the authors' recently described glass electrode method (3) which, it appeared probable, would yield values somewhat more reliable than those obtained by the colorimetric and hydrogen electrode procedures employed by Harris (1) and Levy (2). Acid dissociation constants in formaldehyde are of interest because of their relation to the chemical equilibria involved in formol titrations and their application to practical procedures for the analysis of certain amino acids and peptides in mixtures of these compounds.

EXPERIMENTAL

The purity of the amino acid and peptide samples was established by formol-glass electrode titrations. The modified glass electrode set-up and the experimental technique were those described in the preceding paper (3). One-half an equivalent of standard base was added to the aqueous formaldehyde solution of the amino compounds, the pH of the mixtures determined, and the acid dissociation constants calculated from the expression,

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pH $= \mathrm{pK'}_a$, which was assumed to be valid under these particular conditions.   While percentage impurities revealed by glass electrode analyses were taken into account in calculating $\mathrm{pK'}_a$ values, these corrections were not of appreciable significance, since a 1 per cent deviation in volume of standard base at the mid-point

TABLE I

Summary of Data on Apparent Acid Dissociation Constants of Amino Acids in Water and Aqueous Formaldehyde

Amino acid*	$\mathrm{pK'}_a$			
	Water†	16 per cent HCOH (Harris)	10 per cent HCOH (Levy)	9 per cent HCOH (Authors)
dl-Alanine....................	9.68 (4)	6.4		6.96
l-Aspartic acid...............	3.66 (5)	⪦3.8		
	9.6 (5)	6.85		
d-Glutamic "	4.25 (5)	⪦4.2		
	9.66 (5)	6.8	6.83	
Glycine........................	9.60 (4)	5.4	5.61	5.92
l-Leucine.....................	9.60 (6)		6.83	
dl-Norleucine..................	9.77 (7)			7.10
dl-Phenylalanine...............	9.12 (8)	5.9	6.53	6.80
l-Proline.....................	10.60 (9)		7.73	
dl-Serine.....................	9.15 (7)			5.63
l-Tyrosine....................	9.15 (8)	6.2	7.41	
	10.15 (8)	>9		
dl-Valine.....................	9.64 (8)			7.47

* The optical forms of the amino acids as listed are assumed to be those which Harris and Levy used.   The $\mathrm{pK'}_a$ value, 5.86, for β-phenyl-α-aminoacetic acid in 10 per cent formaldehyde was reported by Levy (2).   However, it is not certain what compound was actually used, since this name appears to be erroneous.

† The $\mathrm{pK'}_a$ values in water were calculated from what seemed to be the most reliable data in the literature.   The figures in parentheses denote bibliographical citations.

of the titrations corresponded to an error of only approximately $0.01\mathrm{pK'}_a$.

A summary of the authors' experimental results is given in Tables I and II.   The constants listed for fourteen amino acids and peptides are the average figures from duplicate determinations for which mean deviations ranging from 0.00 to 0.99 per cent and an average mean deviation of 0.30 per cent were found.   Acid

dissociation constants in formaldehyde and in water from the literature have been included in the tables for purposes of comparison.

Variations in the pK'_a values for a given amino acid, reported by the different authors, may be accounted for by Harris' roughly quantitative generalization that, a fourfold increase of formaldehyde concentration results in 1 unit decrease of pK'_a. While the approximate validity of this hypothesis for glycine has been established in this laboratory by Mr. J. G. Weiner, who employed the authors' glass electrode procedure, no experimental studies on

TABLE II

Summary of Data on Apparent Acid Dissociation Constants of Peptides in Water and Aqueous Formaldehyde

Peptide	pK'_a	
	Water	9 per cent HCOH
Glycylglycine...............................	8.13 (4)	4.27
Diglycylglycine.............................	8.0 (10)	4.24
Glycyl-*dl*-leucine...........................		4.40
dl-Alanylglycine...........................	7.75 (11)	5.52
dl-Leucylglycine...........................		5.55
d-Leucylglycine............................		5.57
l-Leucyl-*l*-tyrosine........................		5.07
dl-Norleucylglycine.........................		5.58

The pK'_a values in water were calculated from what seemed to be the most reliable data in the literature. The values in 9 per cent HCOH are the authors' experimental constants. The figures in parentheses denote bibliographical citations.

other amino acids are available. It is hoped that investigations now under way will throw light on this problem.

It is recognized that the effect of varying formaldehyde concentrations on the various activity coefficients and on the dielectric constant of the medium must be considered. if a strict physicochemical interpretation of the apparent pK'_a values is to be made.

SUMMARY

The apparent acid dissociation constants of a series of amino acids and peptides have been determined with the glass electrode-formol titration method.

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STUDIES ON BIOLOGICAL OXIDATIONS

VI. THE OXIDATION OF PYRUVIC ACID BY GONOCOCCI

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In a preceding communication (1) we have discussed the oxidation of lactic acid to pyruvic acid by α-hydroxyoxidase from gonococci. We presented evidence that this oxidation is performed by the cooperation of two equally important factors: the activating enzyme (Wieland's dehydrogenase), which activates the lactate molecule and renders it ready to be oxidized, and the oxidizing enzyme, which performs the oxidation. In another communication (2) we have shown that the system lactate-enzyme-pyruvate is a sluggish reversible oxidation-reduction system. Its potential was determined by the aid of electromotively active mediators.

This paper will treat of another type of biological oxidation, a type most common and frequently representing the final step in the series of reactions that make up the combustion of foodstuffs. As representative of this type of irreversible oxidation we have taken the oxidation of pyruvic acid by α-ketonoxidase from gonococci. This is an irreversible oxidation, the end-products of which are acetic acid and CO_2, as has been shown by Barron and Miller (3). A comparative study of these two kinds of biological oxidations, reversible and irreversible, has also been made, in an attempt to throw more light on the problem of the mechanism of biological oxidations.

EXPERIMENTAL

Since α-ketonoxidase shows its maximum activity when the bacterial suspensions are freshly prepared from young cultures, we used as the source of this enzyme suspensions of gonococci made from cultures 8 hours old. As the source of α-hydroxyoxidase we

used bacterial suspensions which had been kept in the ice box, at $3° \pm 2°$, for several days to insure complete destruction of α-ketonoxidase. The measurement of the oxygen consumption was made, as usual, at 37°, by means of Barcroft manometers and modified Warburg vessels. The suspensions were buffered with phosphate buffer (0.066 M total phosphate concentration; pH 7.21, unless otherwise stated). Twice recrystallized lithium lactate and pyruvate, in solutions freshly prepared before each experiment, were used as the oxidizable substrate. The other chemicals used were obtained mostly from the Eastman Kodak Company and Kahlbaum. The quinone was recrystallized, and the pyrophosphate purified.

All the figures reported here represent the averages of at least nine experiments.

Rate of Oxidation of Pyruvic Acid and Its Temperature Coefficient

Since Warburg's classic experiments (4) on the inhibiting effect of narcotics on oxidations produced at the surface of charcoal, it has been the general custom of investigators working on biological oxidations to consider enzymic oxidations as surface reactions. We shall present here quantitative evidence in favor of the hypothesis that the oxidation of pyruvic acid by α-ketonoxidase is a surface reaction taking place at the solid-liquid interface, evidence obtained by treating the kinetic data according to Langmuir's conceptions (5).

Let us assume that the oxidation of pyruvic acid takes place at the surface of the enzyme, where the substrate is adsorbed and activated. We are then dealing with a heterogeneous reaction at a solid-liquid interface. Let x be the concentration of substance A in time t; θ, the fraction of the surface covered by the pyruvic acid; then $(1-\theta)$ is the fraction left uncovered. If f is the fraction of A molecules striking the surface and sticking, then the probability of adsorption will be

$$k_1 f x (1 - \theta) \tag{1}$$

the probability of desorption

$$k_2 \theta \tag{2}$$

at equilibrium,

$$k_2 \theta = k_1 f x (1 - \theta) \tag{3}$$

Therefore,

$$\theta = k_1 f x / (k_1 f x + k_2) \tag{4}$$

If we let $f = 1$, divide by k_1, and call k_3 the fraction k_2/k_1,

$$\theta = x/(k_3 + x) \tag{5}$$

The concentration of pyruvic acid at the surface of the enzyme will determine in part both the velocity at which reaction will occur and its apparent kinetic order. Suppose the reaction is proportional to θ, *i.e.* the reaction rate is very small compared with the rate of adsorption and desorption (equilibrium maintained), then

$$- dx/dt = kr(x/(k_3 + x)) \tag{6}$$

where kr and k_3 are constants and x the concentration of pyruvic acid in time t. When the concentration of reactant relative to contact surface is sufficiently great, it is to be expected that the whole of the surface will be steadily and continuously saturated with the reactant, the concentration of which will therefore be constant at the surface. The velocity, being dependent on the number of adsorbed molecules, will be constant, giving a reaction of apparently zero order. This can be seen from Equation 6, for when x is large compared with k_3,

$$- dx/dt = kr \tag{7}$$

As the number of molecules of solute in the bulk of the solution diminishes and the number adsorbed by unit surface falls off continuously and proportionately during the progress of the reaction, the conditions for a unimolecular reaction will be realized, for when x is very small,

$$- dx/dt = (kr/k_3)x \quad \text{(first order reaction)} \tag{8}$$

. In sufficiently dilute solutions, the concentration at the solid surface will not decrease so rapidly as the concentration within the solution. The unimolecular constant will show steadily rising values.

The oxidation of pyruvic acid by α-ketonoxidase of gonococci conformed to these principles governing the kinetics of heterogeneous reactions. Fig. 1, where the rate of oxidation of pyruvic

acid has been plotted, shows the sort of curve which represents the usual adsorption isotherm.

To express the data in a quantitative manner, we have derived
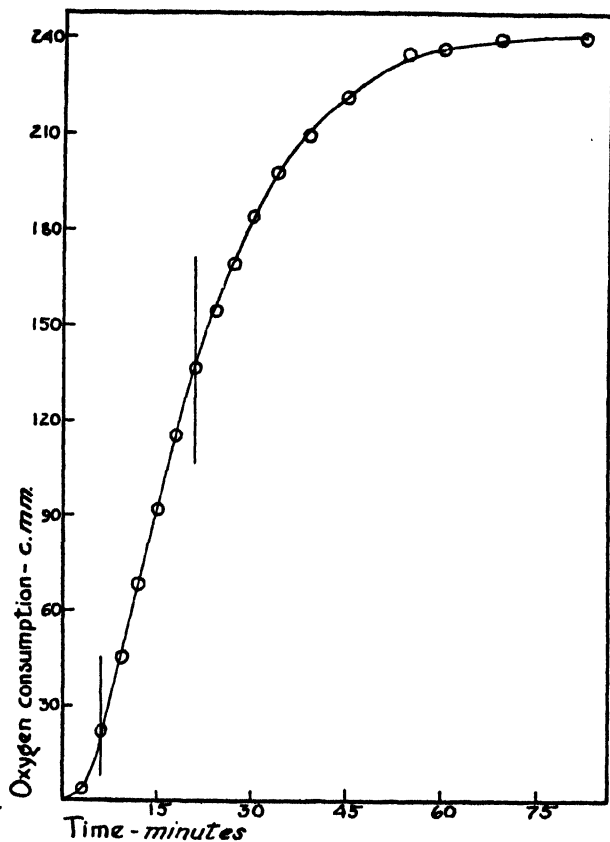
FIG. 1. The rate of oxidation of pyruvic acid by α-ketonoxidase. pH 7.21; T 37°; amount of lithium pyruvate, 0.02 mM.

from Equation 6 a general equation which can be applied to the total length of the reaction. Integrating this equation gives

$$k_3 = (krt - (A - x))/2.3 \log (A/x) \qquad (9)$$

where A is the initial amount of pyruvic acid; x, the amount of pyruvic acid at time t; and kr and k_3 are constants. Giving 6.64

$\times 10^{-5}$ as the value for kr, we have calculated in Table I the values of k_3, values which may be taken as constant in considera-

TABLE I

Rate of Oxidation of Lithium Pyruvate by α-Ketonoxidase

Amount of lithium pyruvate, A, 0.001 mole; T 37°; pH 7.21.

t	krt ($kr = 6.64 \times 10^{-3}$)	$A - x$	$2.3 \log \frac{A}{x}$	$k_3 = \frac{krt - (A - x)}{2.3 \log A/x}$
min.				
2	1.328×10^{-4}	7.32×10^{-5}	0.0750	7.94×10^{-4}
5	$3.32 \ \times 10^{-4}$	1.77×10^{-4}	0.1945	7.97×10^{-4}
8	$5.32 \ \times 10^{-4}$	2.75×10^{-4}	0.3218	7.98×10^{-4}
11	$7.30 \ \times 10^{-4}$	3.77×10^{-4}	0.472	7.50×10^{-4}
14	$9.30 \ \times 10^{-4}$	4.70×10^{-4}	0.634	7.26×10^{-4}
18	1.195×10^{-3}	5.59×10^{-4}	0.817	7.79×10^{-4}
21	1.395×10^{-3}	6.29×10^{-4}	0.989	7.75×10^{-4}
24	1.595×10^{-3}	6.98×10^{-4}	1.195	7.50×10^{-4}
27	1.794×10^{-3}	7.62×10^{-4}	1.433	7.20×10^{-4}
30	1.994×10^{-3}	8.14×10^{-4}	1.68	7.03×10^{-4}
35	2.325×10^{-3}	8.69×10^{-4}	2.03	7.18×10^{-4}
39	$2.59 \ \times 10^{-3}$	9.09×10^{-4}	2.40	7.00×10^{-4}
42	$2.79 \ \times 10^{-3}$	9.24×10^{-4}	2.60	7.18×10^{-4}
46	3.051×10^{-3}	9.46×10^{-4}	2.914	7.22×10^{-4}
54	$3.59 \ \times 10^{-3}$	9.74×10^{-4}	3.64	7.18×10^{-4}

TABLE II

Temperature Coefficient (Q_{10}) for Oxidation of Pyruvic Acid by α-Ketonoxidase

Experiment No.	O_2 consumption in 24 min.		Q_{10}
	At 27°	At 37°	
	c.mm.	*c.mm.*	
1	67.2	207.4	3.08
2	74.5	217.7	2.92
3	65.1	175.9	2.70
4	78.0	218.3	2.80
Average..			2.87

tion of the difficulty of obtaining accurate calculations of oxidation rates from manometric readings where the moving manometers have to be stopped at every reading.

The temperature coefficient (Q_{10}) of this reaction was determined by measuring *simultaneously* the oxygen consumption in two thermostats, one at 27°, the other at 37°. Four series of experiments were performed with three vessels in each series. The concentration of pyruvate was 0.022 M. The pH of the system was 7.00 at 27° and 7.03 at 37°. (At such pH values the activity of the enzyme is at its maximum.) About 6 minutes after the addition of pyruvate, the reaction attained its maximum speed. The rates were compared when the reaction velocity became constant. The average temperature coefficient, Q_{10}, was 2.87 (Table II), which is higher than that reported by Dann (6) for the oxidation of citric acid by "citric dehydrogenase" and that reported by us for the oxidation of lactic acid by α-hydroxyoxidase (2).

Effect of Inhibitors

In the field of enzyme chemistry, where the minuteness of the active catalysts prevents the use of the well known methods of analytical chemistry for discovering their nature, the use of inhibitors and artificial catalysts plays an important rôle. These inhibitors may be arranged in four groups, according to their mode of action: (1) those which combine with the positive catalyst; (2) those which are adsorbed at the surface of the activating enzyme and thus prevent the activation of the substrate; (3) those which in chain reactions interrupt a single process in the chain, thus producing a considerable damping of the velocity by prematurely terminating a chain; (4) those which combine with the oxidizable substrate, forming a compound which is more resistant to chemical change than is the uncombined reactant. In this paper we have studied the first three classes of inhibitors.

Inhibitors of the First Group. Effect of Cyanide—KCN at a concentration of 0.002 M inhibits almost completely the oxidation of lactic acid by α-hydroxyoxidase. At this concentration there was only partial inhibition of the oxidation of pyruvic acid by α-ketonoxidase (77 per cent). It was only when KCN was used at the concentration of 0.01 M that an inhibition of 95 per cent was reached (Table III). We may add that the inhibition produced by HCN on the oxidation of pyruvic acid is not due to cyanhydrin formation.

Effect of H_2S—H_2S at a concentration of 0.002 M inhibits almost completely the oxidation of lactate by α-hydroxyoxidase. In sharp contrast with this inhibiting action, H_2S had almost no effect on the rate of oxidation of pyruvic acid by α-ketonoxidase. The experiments were performed by suspending the bacteria in 0.2 M phosphate at pH 5.2, so that H_2S would be almost completely undissociated. The effect of H_2S on the oxidation of pyruvic acid was measured by determining the production of CO_2. Alkali could not be used as an absorbent of CO_2, for it would have absorbed the H_2S. Because there is no CO_2 production in the oxidation of

TABLE III

Effect of Cyanide, Pyrophosphate, Fluoride, and Hydrogen Sulfide on Oxidation of Lactic Acid and of Pyruvic Acid by α-Hydroxyoxidase and α-Ketonoxidase

Inhibitor	Lactate oxidation			Pyruvate oxidation		
	O_2 consumption in 30 min.		Inhibition	O_2 consumption in 30 min.		Inhibition
	Control	With inhibitor		Control	With inhibitor	
	c.mm.	*c.mm.*	*per cent*	*c.mm.*	*c.mm.*	*per cent*
KCN (0.002 M)................	290	3	99	156	36	77
" (0.01 M)................	137	0	100	235	12	95
$Na_4P_2O_7$ (0.066 M, pH 7.6)......	301	301	None	182	20	89
NaF (0.01 M).................	285	283	"	175	44	75
				CO_2	CO_2	
H_2S (0.002 M, pH 5.2)..........	89	3	97	124	119	4

lactate, in this case the oxygen consumption was measured (Table III).

Effect of Pyrophosphate—Pyrophosphate acts as an inhibitor for the oxidation of cysteine (Warburg and Sakuma (7)), for the oxidation of glutathione (Harrison (8)), and for the oxidation of fructose in concentrated phosphate solutions (Meyerhof and Matsuoka (9)). In all these cases the inhibition is due to combination of pyrophosphate with the positive catalyst, Fe. The oxidation of lactate by α-hydroxyoxidase suspended in either phosphate buffer or pyrophosphate buffer at the same pH and the same molar concentration (pH 7.60; 0.066 M) proceeded with

identical speed.   The effect of pyrophosphate on the oxidation of
pyruvate was quite the reverse.   It inhibited the oxidation to the
extent of 89 per cent, as compared with the rate of oxidation in the
presence of phosphate buffer at the same pH and identical molar
concentration (Table III).   (The pyrophosphate used in these
experiments was Kahlbaum's sodium pyrophosphate, purified ac-
cording to Warburg and buffered with iron-free HCl.)

Effect of Fluoride—It has been shown by Barron and Miller (3)
that NaF at a concentration of 0.01 M has no effect on the oxida-
tion of lactate by α-hydroxyoxidase.   On the other hand, fluoride
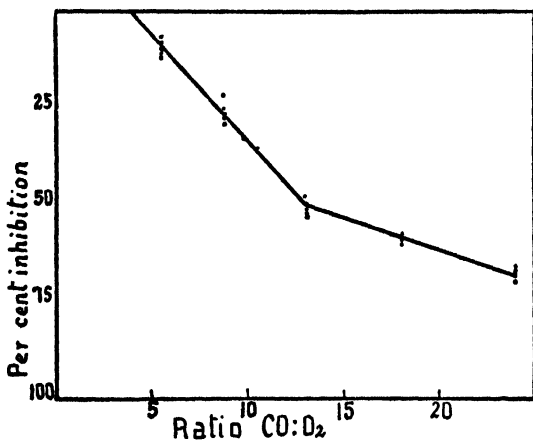
Fig. 2. The effect of CO on the oxidation of lactate by α-hydroxyoxidase.
pH 7.21; T 37°.

at this concentration inhibited the oxidation of pyruvate to the
extent of 75 per cent (Table III).   This inhibiting effect of
fluoride is, we believe, of extreme interest.   Fluoride has a great
affinity for ferric compounds.   The existence of fluoride methemo-
globin was shown by Ville and Derrien (10) and the dissociation
constant of this compound studied by Lipmann (11).

Effect of CO—Cook, Haldane, and Mapson (12) found that the
oxidation of succinate, lactate, and formate by toluene-treated
Bacillus coli showed different degrees of inhibition under the
action of CO.   We have likewise found a difference in the degrees
of inhibition produced by CO in connection with the two catalysts

concerned respectively with the oxidation of lactate and the oxidation of pyruvate. In these experiments CO was prepared by the dehydration of formic acid by H_2SO_4 at 100°. The gas was passed through two washing flasks containing a solution of KOH before it was collected in a container, where the final mixture with oxygen was made. In Fig. 2 we give a summary of the experi-

TABLE IV

Effect of CO on Oxidation of Lactate and Pyruvate by Gonococci
$T = 37°$; pH = 7.11.

Substrate	$r = CO:O_2$	$V = O_2$ consumption in air in 30 min.	$v = O_2$ consumption in $CO:O_2$ mixture in 30 min.	Inhibition	$K = \dfrac{r}{\dfrac{V}{v} - 1}$
		c.mm.	*c.mm.*	*per cent*	
Lactate	8.75	213.0	158.2	25.7	
		211.0	154.7	26.7	
		222.0	160.4	27.8	
		202.7	157.6	22.3	
		201.4	142.6	29.2	
		195.6	143.8	26.5	
Pyruvate	8.10	111.3	113.8	None	
		116.7	114.8	"	
		109.8	112.0	"	
		115.5	113.0	"	
Lactate	13.28	206.8	110.5	46.6	15.3
		193.4	101.2	52.4	14.6
		200.1	101.5	49.3	13.6
		177.0	84.8	52.0	13.2
		190.0	96.3	49.3	13.6
Pyruvate	16.25	204.1	114.1	44.1	20.5
		206.1	112.4	40.8	19.4
		203.2	104.9	48.4	17.3
		174.5	99.7	42.9	21.7
		178.2	102.0	42.8	21.7
		175.3	100.5	42.7	21.8

ments showing the effect of CO on the oxidation of lactate by α-hydroxyoxidase, the abscissa representing the ratio $CO:O_2$ and the ordinate the percentage of inhibition with the control in air taken as 100. Warburg determines the affinity of the enzyme for CO by the use of the equation, $K = r / (V / v - 1)$, in which K is the affinity constant; V, the velocity of oxidation in air; v, the

velocity of oxidation in the presence of $CO:O_2$; and r, the ratio $CO:O_2$. Taking for the determination of K those $CO:O_2$ ratios in which about 50 per cent inhibition was observed, we obtain an average value of 13.7 for the affinity of α-hydroxyoxidase for CO. The affinity of α-ketonoxidase for CO is decidedly lower, the value of K being 20.4. A ratio of 8.7 for $CO:O_2$, which produces an inhibition of 25 per cent on the oxidation of lactate, has no effect on the oxidation of pyruvate (Table IV).

Inhibitors of the Second Group—Warburg (4) has shown in the case of the oxidation of cystine and amino acids by charcoal that alcohols and narcotics act as inhibitors by virtue of the adsorption

TABLE V

Effect of Narcotics on Oxidation of Lactate and of Pyruvate by α-Hydroxyoxidase and α-Ketonoxidase of Gonococci

Narcotic	Lactate oxidation			Pyruvate oxidation		
	O_2 consumption in 30 min.		Inhibition	O_2 consumption in 30 min.		Inhibition
	Control	With narcotic		Control	With narcotic	
	c.mm.	*c.mm.*	*per cent*	*c.mm.*	*c.mm.*	*per cent*
Urethane (0.224 M).............	130.6	91.9	29.6	152.1	19.2	87.4
Phenylurea (saturated)........	312.4	149.9	52.0	413.1	5.6	98.5
Acetonitrile (0.224 M)........	173.6	170.0	None	208.4	154.0	26.0
Valeronitrile (0.05 ")........	136.8	36.5	73.3	208.4	33.9	84.0
" (0.1 M)...........	174.8	6.3	96.5	413.1	7.8	98.0
Valeramide (0.1 M).............	137.8	40.1	71.0	152.1	13.0	91.5

at the surface of charcoal, the degree of inhibition being expressed by Freundlich's equation. It has been found lately that many of these inhibitors (urethanes, alcohols) act also as inhibitors of chain reactions. It is interesting to note that all the inhibitors of this group which have been tested (urethane, phenylurea, acetonitrile, valeronitrile, valeramide) have a more marked effect on the rate of oxidation of pyruvate than on that of lactate, as can be seen in Table V. Octyl alcohol inhibited completely the oxidation of both lactate and pyruvate. The addition of cresyl blue restored the oxidation of lactate but had no effect on the oxidation of pyruvate (Fig. 3). This restorative action of cresyl blue indicates that octyl alcohol inhibits also the oxidizing catalyst of the enzyme,

for the dye can replace the catalyst in α-hydroxyoxidase but not in α-ketonoxidase.

Inhibitors of the Third Group—The induction period shown by the oxidation of pyruvic acid by α-ketonoxidase, the unusually great inhibiting effect produced by narcotics, and the instability of the oxidizing enzyme led us to test the action of inhibitors of chain reactions on the oxidation of pyruvic acid. Our choice of inhibitors was limited by the necessity of their representing as
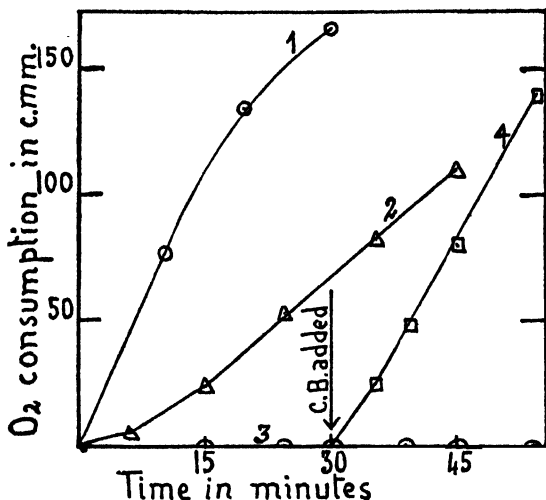
FIG. 3. The effect of octyl alcohol on the oxidation of lactate and pyruvate by their respective enzymes. Curve 1 represents the oxidation of lactate, control; Curve 2, the oxidation of pyruvate, control; Curve 3, the effect of octyl alcohol on the oxidation of pyruvate; Curve 4, the effect of cresyl blue on the oxidation of lactate when the enzyme was inhibited by octyl alcohol.

many different types of compounds as possible and of their being soluble in water. Since the most effective negative catalysts of this class are those substances which are easily oxidized or reduced, we chose quinone, 2,6-dichlorophenol, phenol indophenol, and toluylene blue. All these substances are reduced by the bacterial suspension, and it is the reduced compound which most likely plays the rôle of negative catalyst (Moureu and Dufraisse (13)). We also used p-bromophenol, α-naphthol, dimethylamine, cate-

chol, hydroquinone, pyrogallol, benzyl alcohol, and resorcinol, the chain reaction inhibitors used by Jeu and Alyea (14) in their studies on the oxidation of sodium sulfite. In Table VI we give a summary of the experiments performed with these inhibitors. Quinone was found to be the most effective of all, as a concentration of 1×10^{-5} M was still able to produce an inhibition of 37 per cent. The gradual increase of the inhibiting effect produced by

TABLE VI

Effect of Chain Reaction Inhibitors on Oxidation of Pyruvic Acid by α-Ketonoxidase

Concentration of pyruvate (Na salt) = 0.03 M; pH 7.01; T 37°.

Inhibitor	Concentration of inhibitor	Inhibition
	M per l.	*per cent*
Quinone	0.00003	96
"	0.00001	37
2,6-Dichlorophenol indophenol	0.00003	81
Phenol indophenol	0.00003	64
Toluylene blue	0.00003	58
p-Bromophenol	0.001	87
"	0.0005	61
α-Naphthol	0.001	93
"	0.0005	55
Dimethylaniline	0.001* *Ca.*	97
Catechol	0.001	44
Pyrogallol	0.001	65
Resorcinol	0.005	27
Benzyl alcohol	0.005	19

The per cent inhibition was calculated by comparing the amount of oxygen consumed by the control with that consumed in the presence of the inhibitor. Duration of each experiment, 30 minutes.

* Saturated.

hydroquinone suggests that in reality the inhibition is due to the quinone formed through its oxidation (Fig. 4). The inhibiting effect shown by reversible dyes, especially toluylene blue, is more striking because the same dye acts as a positive catalyst for the oxidation of lactic acid activated by the activating enzyme of α-hydroxyoxidase (Fig. 5). The high inhibition produced by narcotics in the case of pyruvic acid oxidation would be another

indication of a chain reaction mechanism. We must add that this inhibiting effect could also be attributed to adsorption of these substances by the activating enzyme. Our findings, therefore, are offered not as proof of the chain reaction mechanism but as a suggestion that such a mechanism is possible.
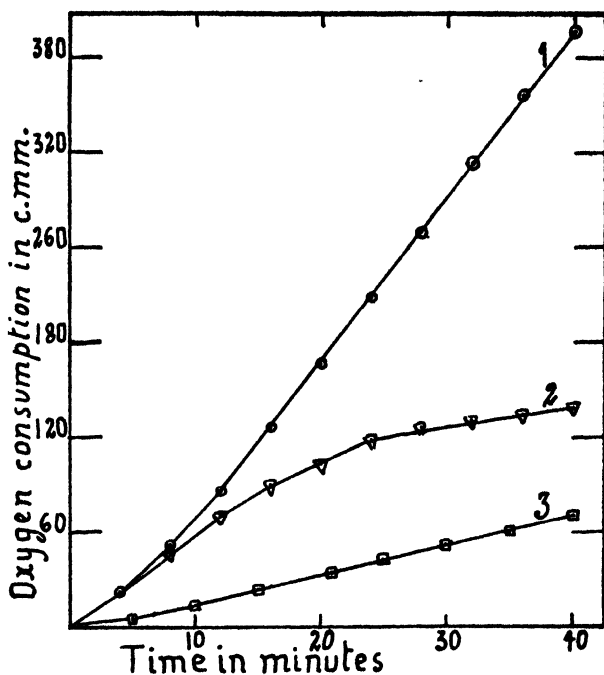
FIG. 4. The effect of quinone and hydroquinone on the oxidation of pyruvate by gonococci. Quinone concentration, 0.01 mM per liter; hydroquinone concentration, 1.0 mM per liter; pyruvate concentration, 30 mM per liter. Curve 1 represents the oxidation of pyruvate, control; Curve 2, the effect of hydroquinone on the oxidation of pyruvate; Curve 3, the effect of quinone on the oxidation of pyruvate.

Stability of α-Hydroxyoxidase and α-Ketonoxidase

It has already been shown that α-hydroxyoxidase retains its activity for as long as 6 months when kept in the ice box, whereas α-ketonoxidase loses its activity quite rapidly. The loss of activity seems to be partly due to the effect of molecular oxygen
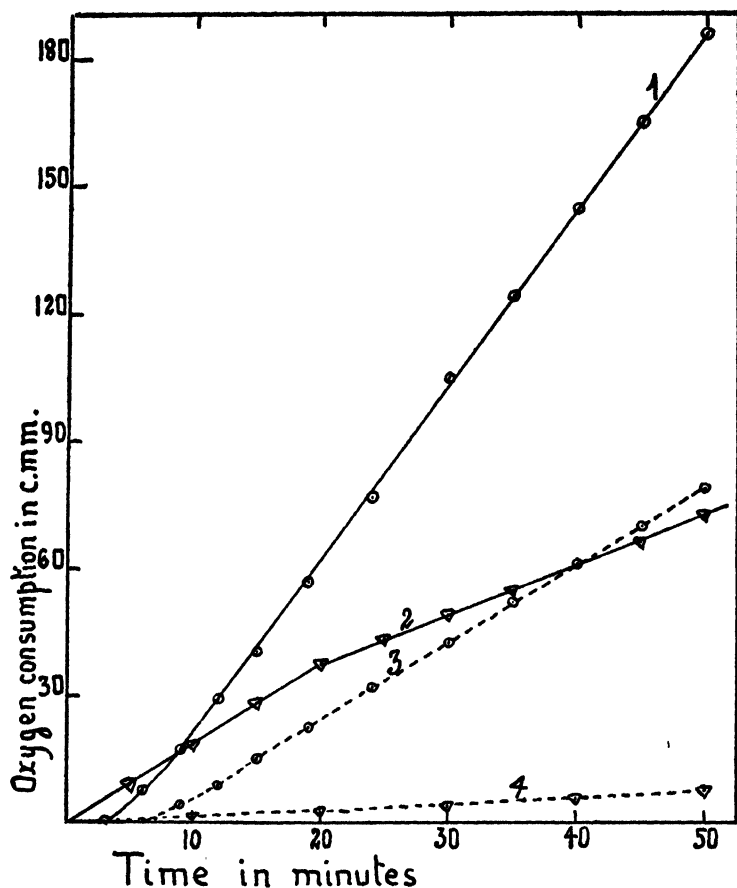
FIG. 5. The effect of toluylene blue on the oxidation of pyruvate and lactate by their respective enzymes. Curve 1 represents the oxidation of pyruvate by α-ketonoxidase, control; Curve 2, the oxidation of lactate activated by the activating enzyme of α-hydroxyoxidase, toluylene blue acting as an oxidizing agent; Curve 3, the effect of toluylene blue on the oxidation of pyruvate; Curve 4, lactate plus the activating enzyme of α-hydroxyoxidase, control.

upon the enzyme. Suspensions of gonococci evacuated for 30 minutes with an oil pump and kept for 24 hours in the ice box retained some active α-ketonoxidase, as tested by the oxidation of pyruvate, although the rate of oxidation had considerably dimin-

ished as compared with the control before evacuation.   When the suspension without pyruvate was shaken in air for half an hour, the enzyme was rendered completely inactive (Table VII).

Remarks on the Activating Enzyme

It has been shown in the case of the oxidation of lactic acid by α-hydroxyoxidase that the lactic acid before being oxidized by the oxidizing enzyme is rendered active, presumably through adsorption at the surface of the enzyme.   We have used the term activating enzyme to distinguish the activation process from the oxidation process.   In the case of the oxidation of lactic acid by α-hydroxyoxidase, it seems that the activated lactate does not leave the enzyme surface until after collision with the oxidizing catalyst, the

TABLE VII

Effect of Molecular Oxygen on Activity of α-Ketonoxidase.   Oxidation of Lithium Pyruvate

Enzyme from	O₂ consumption
	c.mm. per hr.
1. Suspension of gonococci from 8 hr. cultures, used immediately after washing.	197.7
2. Same kept *in vacuo* 24 hrs. in ice box.................	72.0
3. Suspension (1) kept in ice box 24 hrs. in presence of air .	None

oxidation being performed at the surface of the activating enzyme. The following experiments are presented in support of this view.

Experiment I—Protein suspensions were filtered many times through a Berkefeld filter until the filter became impermeable to the activating enzyme of α-hydroxyoxidase.   This filter was inserted into an evacuation flask immersed in a water bath at 38° and connected to a vacuum pump.   The enzyme suspension, buffered with phosphate buffer of pH 7.01 (45 cc. of enzyme suspension in 0.9 per cent NaCl plus 15 cc. of 0.2 m phosphate), was heated to 55° for 1 hour to destroy the oxidizing catalyst.   To 15 cc. of this suspension lithium lactate was added to a concentration of 0.01 m and the suspension put into the filter, which was then stoppered.   The evacuation flask contained 6.5 cc. of H₂O plus 2.5 cc. of 0.2 m phosphate, pH 7.01, plus 0.5 cc. of 0.001 m methylene blue.   The flask was evacuated until most of the liquid in the

Berkefeld filter filtered through; 15 cc. of buffer (45 cc. of H_2O plus 5 cc. of 0.2 M phosphate) were added to the Berkefeld filter, which was stoppered, and filtration under a vacuum was started once more. This evacuation was maintained for 30 minutes without reduction of methylene blue. The lactate which filtered through was, therefore, in the inactive form.

Experiment II—To the fluid in the evacuation flask obtained from Experiment I, 2 cc. of the buffered enzyme were added and the flask evacuated. Methylene blue was reduced in 5 minutes.

Experiment III—The suspension which remained in the Berkefeld filter (6 cc.) from Experiment I was put in the evacuation flask; 3 cc. of water and 0.5 cc. of methylene blue solution were

TABLE VIII

Reduction of Cresyl Blue by Activated Pyruvate

Each tube contained 1 cc. each of enzyme, 0.2 M phosphates, 0.001 M cresyl blue, 0.2 M lithium pyruvate, and inhibiting substance; pH 6.98

Inhibitor	Time of complete reduction
	min.
None...	6
KCN, 0.01 M..	12
NaF, 0.02 "...	6
Pyrophosphate...	13

added. The dye was reduced after 6 minutes of evacuation, showing the presence of activated lactate.

To prove the presence of the activating enzyme in α-hydroxyoxidase, we had inhibited the oxidizing enzyme either by heat or by the action of specific inhibitors, and replaced it with reversible oxidation-reduction systems of suitable oxidation potentials. Such a method could not be used in the case of the oxidation of pyruvic acid, for, when the oxidizing enzyme of α-ketonoxidase was inhibited either by the action of heat or specific inhibitors, oxidation of pyruvic acid did not occur upon the addition of reversible dyes (pyocyanine, methylene blue, cresyl blue, phenol indophenol). The existence of the activating enzyme was demonstrated, however, by the reduction of cresyl blue by active pyruvate in the absence of oxygen. The experiments were performed in test-tubes through which nitrogen was continuously bubbling.

The oxidizing enzyme had been previously inhibited by NaF (0.01 M), KCN (0.01 M), or pyrophosphate (0.066 M). The pH of the system was 7 (Table VIII). In all cases there was reduction of the dye, indicating the concomitant oxidation of the pyruvate. Besides cresyl blue, toluylene blue and methylene blue were also completely reduced, and pyocyanine partially reduced, a fact which would indicate that the apparent reduction potential of pyruvate (Conant and Pratt (15)) lies in the vicinity of the potential of pyocyanine at this pH (-0.034 volt). Indigodisulfonate (-0.125 volt) was not visibly reduced.

<div align="center">DISCUSSION</div>

Numerous investigators have brought out evidence that biological oxidations are surface reactions. We may recall Warburg's observations on the inhibiting effect of narcotics on the oxidation of amino acids by his charcoal model, where the degree of inhibition was related to Freundlich's adsorption isotherm; and Penrose and Quastel's experiments (16) on the effect of lysis on the activity of the oxidation enzymes of *Micrococcus lysodeikticus*. If biological oxidations are surface reactions—*i.e.*, heterogeneous reactions taking place at the solid-liquid interface—the rate of reaction may then be formulated by equations derived from the Langmuir adsorption isotherm (5). In the experiments here reported the oxidation of pyruvic acid by α-ketonoxidase to acetic acid and CO_2 was taken as a model to test the validity of the hypothesis, because this oxidation is an irreversible process in which the end of the reaction can be easily reached. The fairly close agreement found between the rate of oxidation and the general equation derived from Langmuir's adsorption isotherm, in spite of technical difficulties, is to us a quantitative proof that this oxidation can be treated as a heterogeneous reaction. We have also presented qualitative evidence similar to that offered by Warburg, namely, the inhibiting effect of narcotics on the speed of oxidation, a degree of inhibition which is high when compared with the inhibition produced by such narcotics on the oxidation of lactic acid by α-hydroxyoxidase. We shall refer later to the probable meaning of this high inhibiting power of narcotics.

In the case of the oxidation of lactic acid by α-hydroxyoxidase it was possible to separate the activating enzyme (which renders the

lactate molecule easily oxidizable) from the oxidizing catalyst (which performs the oxidation process) and replace the latter by artificial catalysts. In the case of the oxidation of pyruvic acid, the lack of a suitable oxidizing catalyst made such separation impossible. Evidence of activation of the pyruvic acid was obtained in experiments performed in the absence of oxygen; in these experiments pyruvic acid in the presence of the enzyme was oxidized by reversible dyestuffs of proper oxidation-reduction potential. Since pyruvic acid alone is not oxidized by such mild oxidative agents, activation of the molecule by the enzyme is indicated.

The rôle of the activating enzyme is, as we have already stated, to render the substrate accessible to the action of the oxidizing catalyst. This is the enzyme called dehydrogenase by Thunberg and Wieland. That such activation is performed at the surface of the activating enzyme has been shown by the inhibiting effect of narcotics on the oxidation of activated lactic acid performed by either the normal cellular catalyst or the artificial catalyst (2). The active molecule seems to remain adsorbed at the surface of the activating enzyme, as has been shown in experiments with lactic acid and α-hydroxyoxidase. The formation of such activated molecules has been postulated by Bodenstein (17) as the initial step in the oxidation of benzaldehyde, where the activated aldehyde reacts with oxygen, giving an unstable peroxide and thus starting the chain reaction. Whether this activated molecule may be considered a free radical, a hypothesis postulated by Haber and Willstätter (18) and embodied in Bodenstein's theory, remains to be proved.

The opinion that there are specific enzymes for the activation of every substrate is not advanced now, even by the supporters of the pluralistic theory, as such a view would lead us to accept the existence of at least 56 enzymes in a cell the size of *Bacillus coli*. (Quastel and Whetham (19) found that this cell is able to oxidize 56 substrates.) The possibility of obtaining enzymes which will activate a determined chemical group of substrates cannot be denied. Stephenson's lactic dehydrogenase (20), Bernheim's lactic and citric dehydrogenases (21), our α-hydroxyoxidase (2), and Thunberg's succinodehydrogenase (22), have, among others, been reported as acting upon a limited number of chemically

allied substances. The possibility of separating these group-specific enzymes outside the cell may be explained by applying to biological oxidations the prevailing theories regarding catalytic heterogeneous reactions (Quastel's theory (23) is essentially such an application). It is plausible to postulate that there exist at the surface of the cell selective active centers where activation of the substrate occurs. This theory has in its favor many examples of surface catalytic reactions where the existence of independent centers of activity on the catalyst surface with different modes of adsorption has been demonstrated. Taylor (24) has expressed the view (supported by several examples from various investigators) that only a small fraction of the total surface is necessarily active in catalyzing a given reaction, and, moreover, that this fraction is a function not merely of the surface itself but of the reaction catalyzed. The centers which are active in respect to one reaction may be quite inactive in respect to another, although the surface regarded as a whole catalyzes both equally well. Adkins and Nissen (25) found that alumina prepared in different ways exhibited decidedly varying catalytic activity toward the decomposition of formic acid. Moreover, the apparent increments of energy varied from one kind of alumina to another. The actual spacing of the atoms in the solid catalysts has, according to several investigators (see Langmuir (26)), a very pronounced influence on the catalytic activity. The idea that surfaces of heterogeneous catalysts present regions of graded activity is certainly an aid in the interpretation of biological activation.

The oxidizing catalyst in the case of pyruvic acid oxidation has been shown to be different from that of lactic acid oxidation. The fact that the oxidation is inhibited by pyrophosphate, fluoride, and cyanide, makes us favor the opinion that the oxidizing catalyst is a ferric complex compound. Whether this ferric complex catalyst is a hemin derivative we do not know. The mechanism of this oxidation still remains in the speculative stages. The induction period observed in the oxidation of pyruvic acid by α-ketonoxidase, the high inhibiting effect produced by narcotics which are considered among the inhibitors of chain reactions, and the inhibiting effect produced on this oxidation by a series of other substances known to act as inhibitors in chain reactions suggest the possibility that this oxidation is a chain reaction starting with the activation

of the molecule at the surface of the enzyme. Recently a considerable body of experiments has been presented showing that many oxidation processes are chain reactions. The oxidations of acetaldehyde (Bodenstein (17); Backström (27)), of alkali sulfite (Alyea and Backström (28)), of FeSO₄ (Richter (29)), of saturated hydrocarbons (Pease (30)), of methyl alcohol and formaldehyde (Fort and Hinshelwood (31)), and of H₂S (Thompson and Kelland (32)) have been shown to conform to the theory of chain reactions. The action of "stabilizers" in preserving fatty acids and carotene from oxidation also suggests that these oxidations may all have the same chain reaction mechanism.

SUMMARY

The oxidation of pyruvic acid by α-ketonoxidase is performed by: (1) activation of the molecule by the activating enzyme; (2) its oxidation by the oxidizing enzyme. The temperature coefficient, Q_{10}, for the oxidation of pyruvic acid by α-ketonoxidase was found to be 2.83. The rate of reaction conforms to the supposition that the oxidation is a heterogeneous reaction taking place at the solid-liquid interface. The induction period of the reaction and the inhibiting effect produced by a series of substances known to be chain reaction inhibitors suggest the possibility that the oxidation of pyruvic acid is a chain reaction. A comparative study of the action of inhibiting agents on the oxidation of lactic and pyruvic acids by α-hydroxyoxidase and α-ketonoxidase shows that these two enzymes are not identical.

The mechanism of activation and oxidation of biological oxidations has been discussed.

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INFLUENCE OF CERTAIN DYESTUFFS ON FERMENTATION AND RESPIRATION OF YEAST EXTRACT

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Dyestuffs which form reversible oxidation-reduction systems have been used in biology chiefly as indicators (1) of oxidation-reduction potentials and as stimulants for respiration (2). Some of these dyes are also known to affect the fermentation reactions of certain living cells under both aerobic and anaerobic conditions (3). In the case of oxygen consumption the dyes are believed to act either as supplements to or in place of naturally occurring respiratory enzymes. In those cases where the fermentation reactions are influenced, it is not known how the effect is brought about or at what stage of the fermentation cycle the effect is produced. In view of the fact that the study of cell-free yeast extracts has appreciably advanced our knowledge of carbohydrate metabolism, chiefly because the intermediate steps are more accessible than in living cells, it seemed desirable to investigate the influence of such dyes on such extracts. These extracts actively ferment glucose to alcohol and CO_2. They consume only small amounts of oxygen and their fermenting activity is practically independent of the presence or absence of oxygen. If, however, a dyestuff that can be reduced by the extract and oxidized by oxygen is added to such a system, an appreciable oxygen consumption takes place. The problems we have studied are, what effect do these dyes have on the fermenting activity of the extract, is this effect related to the oxygen consumption, and at what stage is the effect exerted?

According to Lipmann (4) who has reported results with two dyes on such extracts, the oxygen consumption brought about by such a dye may or may not inhibit fermentation. He concludes

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that the deciding factor is the oxidation-reduction potential which the dyestuff imposes on the fermenting mixture. If the potential range of the dye added is relatively negative, no inhibition occurs. If it is relatively more positive and oxygen is supplied at a rate sufficient to keep a part of the dye in the oxidized form, inhibition does occur and can be reversed if the potential is lowered. He lowered the potential by making the experiment anaerobic, and adding ascorbic acid as a reductant. In addition, however, it should be noted that he always also added hexosediphosphate at the same time. He regards this reversible change as an example of the Pasteur reaction and considers the mechanism of this reaction to be the reversible oxidation of some component of the fermenting system which is active only in the reduced form. Whatever the nature of the Pasteur reaction may prove to be, we believe the interpretation that the effect of a dyestuff on these extracts is dependent on the potential which it imparts to the mixture is not in keeping with our results.

When we speak in what follows of the potential of the extract, we mean the potential as established at an electrode without implying that it is justifiable to speak of a potential in a strictly thermodynamical sense in a system the components of which are not in equilibrium with each other. The potential as measured at the electrode depends to a very large extent on the rate at which oxygen is supplied. The degree of decolorization of added dye indicators will roughly agree with the electrode measurements because the electrode responds more easily to the rapidly reacting systems such as the added dyestuffs than to the others. Consequently, just as the electrode potential varies with the rate of oxygen supply, the degree of decolorization of an indicator also varies with this rate of supply. Our potential measurements are comparable only in so far as they are performed at approximately the same partial pressure of oxygen and are not meant to represent precise and strictly reproducible values nor do they represent the potential of the whole system in a thermodynamical sense.

Preparation and Properties of Yeast Extract—The yeast used was a bottom yeast kindly supplied to us by Ruppert's brewery. It was pressed at 800 pounds pressure, dried in the air at 25°, ground to a fine powder, and stored in the ice box. The extract was prepared from a portion of this powder with 3 volumes of distilled water

according to von Lebedev (5).   Various samples had a pH of 6.10, varying no more than ±0.05 pH unit, measured with the glass electrode, and were slightly less than 0.1 M in inorganic phosphate. When very fresh, 1.0 cc. of this extract with 0.1 gm. of glucose, when shaken in the usual Warburg manometric apparatus at 37°, produced CO_2 at a rate of 200 c.mm. per minute during the rapid phase of the fermentation.   For the convenience of the readings the extracts were always diluted in the experiments.   On standing at room temperature the extract gradually looses its activity, and as used in some of the experiments shows a considerably smaller

TABLE I

Dyes Studied

	Dye	Biblio-graphic reference No.	Group	Normal potential at pH 6.0
				volt
1	1-Naphthol-2-sulfonate indophenol	6,7	B	+0.183
2	Thionine	6,7	C	+0.100
3	Gallocyanine	7	A	+0.080
4	Pyocyanine	7	C	+0.050 (Second step)
5	Methylene blue	6,7	"	+0.047
6	Indigodisulfonate	6,7	"	−0.069
7	Gallophenine	7	"	−0.077
8	Brilliant alizarin blue	7	B	−0.112
9	Phenosafranine	8	A	−0.218
10	Rosinduline GG	7	B	−0.220
11	Neutral red	9	A	−0.271

activity than that quoted.   All of the extracts used in these experiments were free of self-fermentation; *i.e.*, they did not produce CO_2 before substrate was added.

All the extracts prepared exhibited strong reducing properties. Anaerobically methylene blue and indigodisulfonate were completely and rapidly reduced.   Even rosinduline GG was completely, although very slowly, reduced.   As judged by the iodine titration, oxidized glutathione was reduced at an appreciable rate. Benzyl viologen was not reduced.

Dyestuffs Used and Their Classification According to Their

Effect—The dyes, arranged in Table I in the order of their normal potentials at pH 6.0, were tested for their effect on oxygen consumption and fermentation. With the exception of rosinduline GG, they all brought about an appreciable oxygen consumption. In mammalian erythrocytes (2) and in certain tissues the more negative dyestuffs of this series have no effect on respiration. This behavior is in agreement with the fact that yeast extracts, quite generally, show a much stronger reducing faculty than blood or than most tissues. Dyestuffs more positive than those mentioned, such as phenolindophenol, are kept completely reduced by the yeast extract because they are only slowly oxidized by oxygen under these conditions.

The general effect of small amounts of dyes on fermentation is to shorten the induction period provided some hexosediphosphate is present, and to accelerate the beginning stages. Under anaerobic conditions this is the only effect of small amounts of dye, but larger amounts may distinctly inhibit, although there may be an initial period of acceleration. None of the dyes tested stops anaerobic fermentation entirely, but rosinduline GG almost does so. Under aerobic conditions oxygen is consumed and the effect on fermentation varies from almost complete inhibition to no inhibition at all. This inhibition may in some cases be preceded by a marked acceleration. The order of the dyes arranged according to their inhibiting effect under the conditions given in Figs. 1 to 9 is as follows: rosinduline $\lessgtr$ naphtholsulfonate indophenol > pyocyanine > brilliant alizarin blue > indigodisulfonate > gallophenine > thionine = methylene blue > phenosafranine > gallocyanine > neutral red. It is evident that this order is not the same as the order of the normal potentials.

The order with respect to the effect on oxygen consumption is much more difficult to give. This effect varies with concentration of the dye as well as with temperature, and the different dyes do not vary in the same way. Sufficient data for our present purpose are given in Figs. 1 to 8.

Three of the dyes (Samples 3, 9, and 11, Table I) show practically no inhibition, although they bring about an appreciable oxygen consumption. They have been classed as Group A in Table I, and require no further consideration. The three dyes of Group B bring about an inhibition that, as will be described in

detail, can be readily overcome by addition of hexosediphosphate. These dyes may be considered as specific poisons for the enzyme or the system of enzymes responsible for the synthesis of hexose-diphosphate. Their effect seems to correspond to what Lipmann considered as a reversible inhibition. The remaining dyes, Group C, although they differ widely from each other in quantitative effect, seem to be alike to the extent that the inhibition which they cause is entirely irreversible and due to the destruction of enzymes. Their inhibition could not be overcome by addition of any of the intermediate substrates tested.

<div align="center">EXPERIMENTAL</div>

Groups A and C—Fig. 1 shows some results with pyocyanine, Fig. 2 some with thionine, Fig. 3 some with indigodisulfonate, all of Group C, at 37°. Fig. 4 shows some results with four dyes of this group and one of Group A, gallocyanine, at 28°. The lower part of Fig. 4 shows the oxygen consumption and the upper part the total CO_2 production. On comparison of Figs. 1 and 2 it is evident that pyocyanine inhibits more strongly than thionine, and that the oxygen consumption at the higher concentration is about the same for the two dyes. On attempting to explain the differences of these two dyes one may first attempt to seek a correlation to potentials as claimed by Lipmann. One may first think of the normal potentials of these dyes. Then, the order, as regards their inhibiting effect, is just the reverse of that claimed by Lipmann. In the second place, one may think of the actual potential which is imposed on the yeast extract on addition of the dye. Both electrode measurement (Fig. 9) and observation of reduction of the dye from the degree of decolorization show that the potential is not more positive with pyocyanine than with thionine. In the same way, the effect of indigodisulfonate and the lack of effect of gallocyanine (Fig. 4) cannot be explained on the ground of potentials, or of the oxygen consumption brought about by the dyes. Whether the strong effect of pyocyanine is causally connected with its faculty of semiquinone formation cannot be definitely decided at the present time.

The inhibition by pyocyanine is not reversed by making the experiment anaerobic and adding a reductant such as ascorbic acid, together with hexosediphosphate. This is true for all the
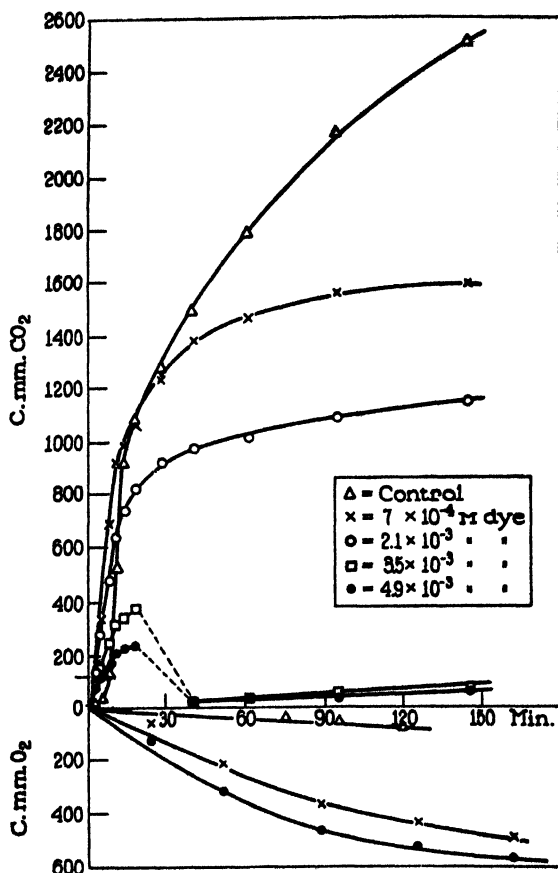
FIG. 1. The effect of pyocyanine at 37°. The experiments were carried out with the usual Warburg manometric technique. The contents of each vessel were as follows: The main room contained 0.5 cc. of extract and the amount of dye necessary to give the final concentration shown, in a total volume of 1.2 cc. A side arm contained 0.2 cc. of 40 per cent glucose and 0.02 cc. of 0.15 M potassium hexosediphosphate. The contents were mixed at time 0. The gas room contained air. In the oxygen consumption experiments 0.2 cc. of 10 per cent NaOH were added to the inset. The dotted lines represent a change to anaerobic conditions. In each experiment (represented by an individual curve) 0.02 cc. of 0.15 M potassium hexosediphosphate and 1.0 mg. of ascorbic acid dissolved in 0.1 cc. were added after the conditions were made anaerobic. The vessels were shaken at the rate of 60 times per minute through a distance of 5 inches.

dyes of Group C. If the change to anaerobic conditions is made when the fermentation is only partly inhibited, it continues at the reduced rate and does not return to the control rate.
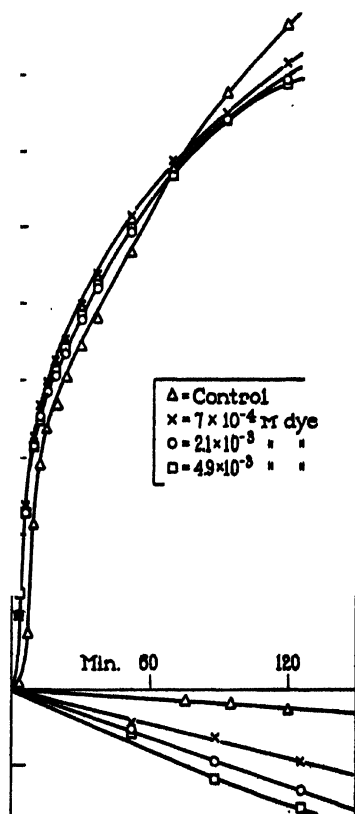
FIG. 2

FIG. 3

. FIG. 2. The effect of thionine at 37°. The contents of each vessel were as given for Fig. 1.
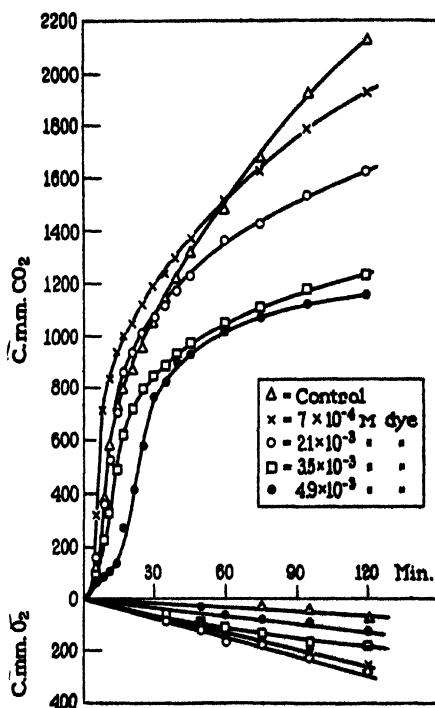
FIG. 3. The effect of indigodisulfonate at 37°. The contents of each vessel were as given for Fig. 1.

It can be shown that the cause of the cessation of fermentation is the destruction of enzymes. This follows from the following

observation.   The fermentation, after its cessation, can be restored by addition of fresh yeast extract, but not of extract previously heated at 80° (Fig. 5, Curves A and C).   Certainly not all enzymes are destroyed, for the reducing power of the extract is still intact after the fermentation has stopped.   Even the largest
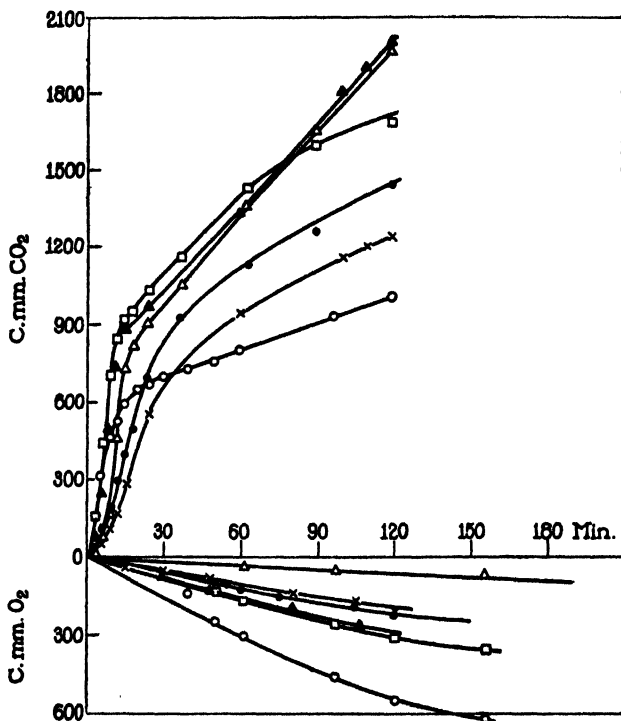
FIG. 4. The effect of five dyes at 28°.   The contents of each vessel were as given for Fig. 1. $\triangle$ represents control; $\square$, 4.9×10^{-3} M thionine; $\bullet$, 4.9×10^{-3} M indigodisulfonate; $\times$, 4.9×10^{-3} M gallophenine; $\circ$, 4.9×10^{-3} M pyocyanine; $\blacktriangle$, 4.9×10^{-3} M gallocyanine.

amounts of pyocyanine were reduced in about 10 minutes after the conditions were made anaerobic at 37°.   At lower temperatures the reduction was slower.   Addition of ascorbic acid is not necessary to restore the anaerobic reducing faculty.

It could be definitely shown that carboxylase is one of the en-

zymes destroyed by these dyes—for pyruvic acid (adjusted to the pH of the extract) is not decarboxylated by extracts treated with dye, although it is rapidly decarboxylated by normal extracts. It could also be directly shown that carboxylase is very sensitive to treatment with these dyes. A few attempts at purifying
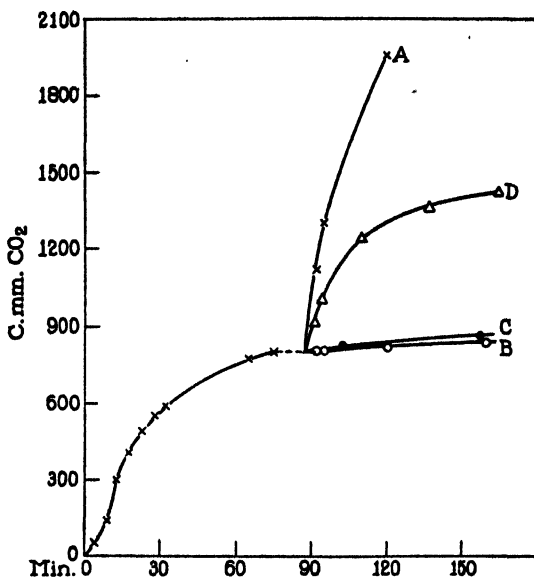
FIG. 5. The effect of various preparations in restoring fermentation to an inhibited extract. The contents of four vessels made up as for Fig. 1, each containing 3.5×10^{-3} M pyocyanine, were run at 37° for 75 minutes, as shown in the first part of the curve. The conditions for each were then made anaerobic. To the vessel represented by Curve A was added 0.5 cc. of fresh extract; to that by Curve B, 1 mg. of ascorbic acid and 1.2 mg. of potassium hexosediphosphate in 0.1 cc. of solution; to that by Curve C, 0.5 cc. of an extract that had been heated to 80° and filtered; to that by Curve D, 0.5 cc. of a 5.0 per cent carboxylase preparation.

carboxylase are recorded in the literature. One of such preparations obtained by precipitation with acetone according to Axmacher and Bergstermann (10) was tested and found to be very active toward pyruvic acid without being able to ferment glucose, even after addition of heated extract. Such a carboxylase preparation was able to restore the fermentation of the extracts inacti-
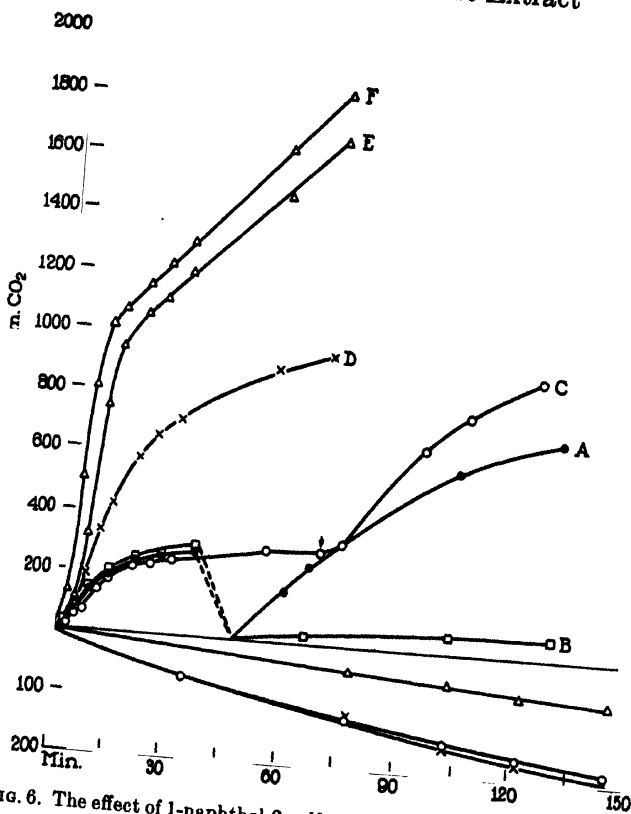
FIG. 6. The effect of 1-naphthol-2-sulfonate indophenol at 28°.   Curve E represents the aerobic control, as for Fig. 1.   The experiments represented by Curves A, B, and C are the same as that for Curve E with the addition of 4.9 × 10⁻³ M dye. After 37 minutes the conditions in the experiments represented by Curves A and B were made anaerobic. 1 mg. of ascorbic acid (half neutralized) was added in the experiment represented by Curve B. 0.1 cc. of 0.15 M potassium hexosediphosphate was added in the experiment represented by Curve A. At 69 minutes, shown by the arrow, 0.1 cc. of 0.15 M potassium hexosediphosphate was added in the experiment represented by Curve C under aerobic conditions. Curve F represents an aerobic control to which 5 times the usual amount (i.e. 0.1 cc. of 0.15 M) of diphosphate was added at the beginning. The experiment represented by Curve D is the same as those for Curves A, B, and C, except that the amount of diphosphate added at the beginning is 5 times as great (i.e. equal to that in Curve F). The oxygen consumption shown is for the experiments represented by Curves C and D. It should be noted that the scale for oxygen is different from that for CO₂.

vated by the dye (Fig. 6, Curve D). The carboxylase preparation itself was rapidly inactivated by treatment with the dye in the presence of oxygen. It cannot, however, be claimed that carboxylase is the only enzyme destroyed by the dye. First of all, it is quite sure that such a carboxylase preparation contains other enzymes. Furthermore, if no other enzyme than carboxylase had been destroyed, we should have been able to find pyruvic acid in extracts inactivated by the dye. It could be shown that an acid is produced under these conditions, for the pH of the extract, originally 6.01, after shaking with the dye for 45 minutes at 37° had fallen to 5.20 (measurement with glass electrode). This increase in acidity is not sufficient to explain the cessation of fermentation. This acid was apparently not pyruvic acid for attempts to isolate it as the phenylhydrazone were unsuccessful. Either pyruvate had been changed in some way other than by decarboxylation or the inhibition of fermentation occurred at some stage prior to pyruvate formation.

The acid produced was not lactic acid. Our extracts always contained small amounts of lactic acid (determined by the method of Friedemann, Cotonio, and Shaffer (11)), but this amount, which was not changed during normal fermentation, consistently decreased in the dye experiments. It appears that the lactic acid present is one of the substrates oxidized.

The behavior of the inorganic phosphates can be described as follows. If the fermenting power of the extract is relatively low and the inhibition by the dye is quickly established, the fermentation may entirely cease before the inorganic phosphate has changed very much. If, however, the fermenting power is high and the inhibition by the dye only slowly established, the inorganic phosphate may have decreased to very low values by the time the fermentation stops. After the fermentation stops, the oxygen consumption continues for some time, although it gradually declines, but this is not accompanied by a change in inorganic phosphate. Added inorganic phosphate remains as such and added hexosediphosphate is not hydrolyzed to inorganic phosphate. The addition of acetaldehyde does not restore the phosphorylation, but when the fermentation is restored by the carboxylase preparation phosphorylation is, of course, also restored. This together with the other evidence indicates that some enzyme which acts

before the pyruvate stage is reached is also destroyed. To summarize, the effect of the dyes of Group C is irreversible. It is due to the destruction of some enzymes, among which carboxylase can be definitely recognized as one.

Group B—The dyestuffs of Group B behave quite differently. When an extract is treated with naphtholsulfonate indophenol[1] at 28° under the same conditions as with the other dyes, the fermentation is almost entirely inhibited. If the air is then replaced with nitrogen and ascorbic acid plus hexosediphosphate added, as described by Lipmann, the fermentation is, at least to a large extent, restored (Fig. 6, Curve A). A similar effect is obtained at 37°, but here the irreversible effect described above also comes into play. According to our results the ascorbic acid is entirely unnecessary for this effect, the diphosphate alone being sufficient. This is not quite unexpected as the reducing faculty of the extract is still intact and need not be supplemented by ascorbic acid. However, one should not conclude from this statement, that the reduction is necessary for restoring the fermentation, for the fermentation returns before any appreciable reduction of the dye has taken place. On the other hand, the reduction of the dye without the addition of diphosphate does not restore fermentation (12) (Fig. 6, Curve B). Furthermore, addition of diphosphate under aerobic conditions does restore the fermentation temporarily (Fig. 6, Curve C), and under these conditions the dye does not show any marked change of color indicating an appreciable reduction. The potential measurements with a bright platinum electrode agreed essentially with the observations of the color. To be sure, after adding diphosphate, a drop of from 10 to 12 millivolts occurred almost instantaneously, but after 2 minutes the potential began to rise again and rapidly attained its former value. The fermentation then continued at this positive potential level. Similarly, the inhibition brought about by this dye is greatly delayed by the addition of larger amounts of

[1] A commercial sample of 1-naphthol-2-sulfonate indophenol did not give the results described here, but brought about a rapid and irreversible inhibition. The titration curve of this sample indicated it to be very impure. The results reported here were obtained on a product synthesized in this laboratory, which, according to a potentiometric titration, was pure and agreed in its normal potentials with the value reported by Clark and Cohen (6).

diphosphate with the glucose at the beginning (Fig. 6, Curve D), but the potential is not kept at a more negative level than when less diphosphate is added.

It appears, therefore, that this inhibition is brought about by the removal of the hexosediphosphate, or some products normally derived from it, and the blocking of the reaction by which this substance is normally formed. It has been known for some time that diphosphate acts as a catalyst in abolishing the induction period in yeast extracts, and its rôle in the chain of reactions that constitutes fermentation is now believed to be known (13).

Acetaldehyde, pyruvate, and adenylpyrophosphate are entirely unable to restore this fermentation. Hexosemonophosphate has a very slight activity, but is not comparable with hexosediphosphate. The restored fermentation is not, however, just the fermentation of the added diphosphate, for it is not only much more rapid but also goes far beyond this in amount if the experiment is made anaerobic to prevent or at least hinder the recurring of the inhibition, and it is accompanied by a disappearance of inorganic phosphate from the solution. It appears then that this dye in the presence of oxygen prevents the formation of diphosphate from glucose and inorganic phosphate and also its formation by the cycle of reactions by which the phosphorus of added hexosediphosphate is converted eventually into more hexosediphosphate. If now, after it has acted upon the extract, the dye is reduced, the ability to form diphosphate from glucose and inorganic phosphate is not restored, but the ability to complete the cycle from added hexosediphosphate to more diphosphate is at least in part restored. Thus the inhibition is in part reversible and in part non-reversible. In terms of Meyerhof and Kiessling's most recent equations (13) this would mean that the transfer of phosphate from the phosphopyruvic acid to the glucose is not inhibited by either the oxidized or reduced form of the dye, since the fermentation of the diphosphate added is rapid, but the mechanism by which this acceptor finally forms diphosphate is inhibited by the oxidized form of the dye and not inhibited or at least inhibited to a smaller extent by the reduced dye. However, the mechanism by which diphosphate is normally formed during the induction period is inhibited by the oxidized dye and is not restored by reducing the dye.
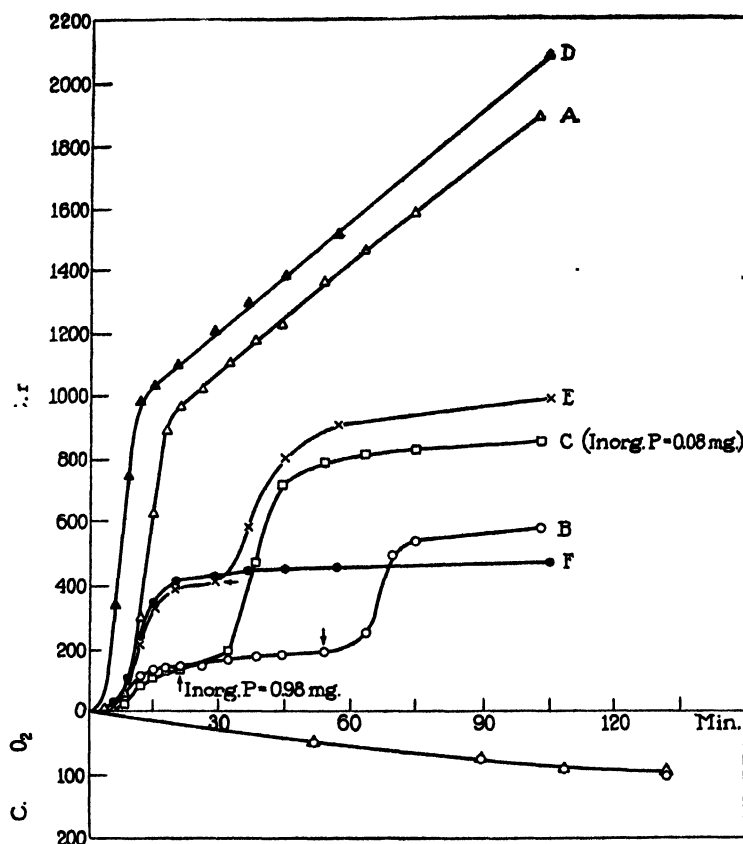
FIG. 7. The effect of rosinduline at 28°. Curves A, B, and C represent experiments performed under aerobic conditions. Curve A represents the control without dye. The contents are as given for Fig. 1. Curves B and C are for samples 4.9×10^{-3} M with dye. At 21 minutes 0.1 cc. of 0.15 M potassium hexosediphosphate was added in the experiment represented by Curve C. At 54 minutes 0.05 cc. of diphosphate was added in the experiment represented by Curve B. Curves D, E, and F represent experiments performed under anaerobic conditions. Curve D represents the control, but with more diphosphate (0.1 cc. of 0.15 M) than in the experiment represented by Curve A. The experiments represented by Curves E and F are like that of Curve D but in addition the samples are 4.9×10^{-3} M with dye. At 29 minutes 0.1 cc. of diphosphate was added in the experiment represented by Curve E. The lower part of the figure shows that 4.9×10^{-3} M rosinduline does not increase the oxygen consumption beyond that of the control. The two figures for inorganic phosphate given on Curve C show how this decreases upon the addition of diphosphate.

From the above discussion it is evident that, since diphosphate is not formed in the presence of the dye and yet inorganic phos-
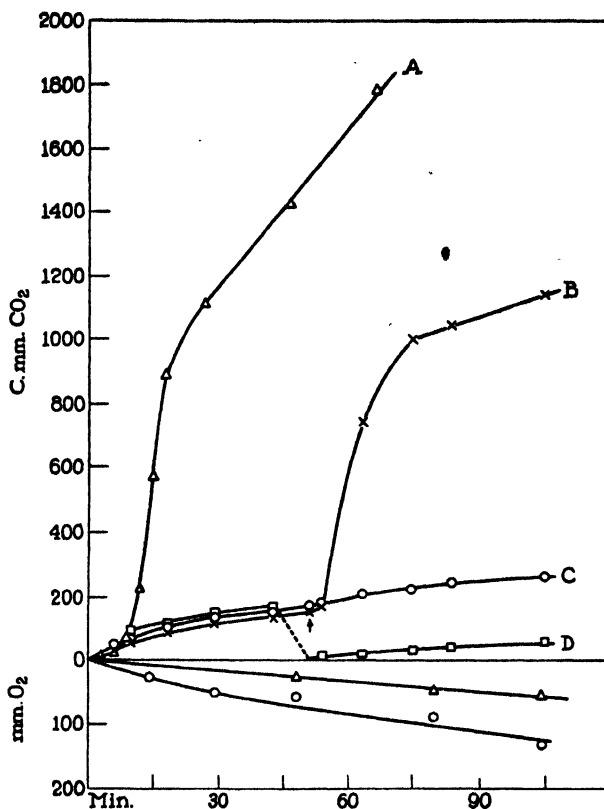
FIG. 8. The effect of brilliant alizarin blue at 28°. Curve A represents the control without dye. Curves B, C, and D are for samples 4.9 × 10⁻³ M with dye. At 43 minutes the conditions of the experiment represented by Curve D were made anaerobic and 1 mg. of ascorbic acid (half neutralized) was added. At 51 minutes, indicated by the arrow, 0.1 cc. of 0.15 M potassium hexosediphosphate was added in the experiment represented by Curve B. The oxygen consumptions shown are for the experiments represented by Curves A and C.

phate disappears, the phosphate must be stored up in some other organic form. What this substance is will be shown in a subsequent paper.

Rosinduline GG brings about the same effect as naphtholsulfonate indophenol.   These two dyes differ widely in their normal
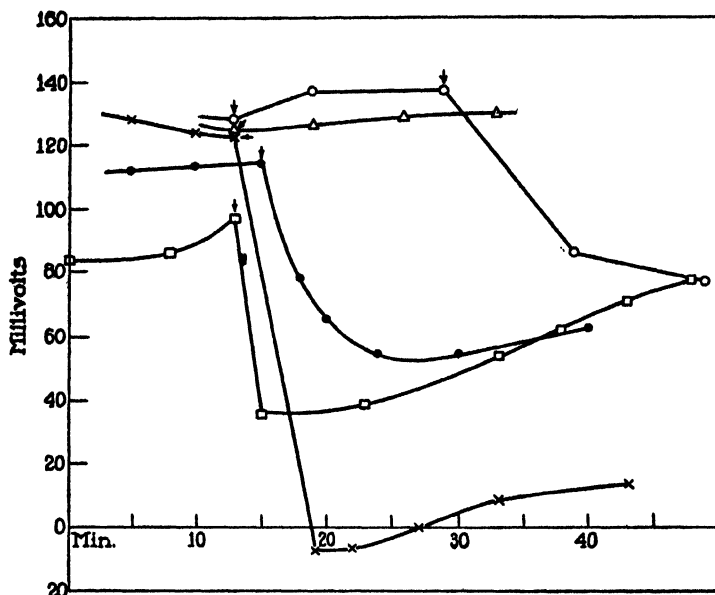
FIG. 9. Some potential measurements on extracts containing various dyes.   The potentials were measured against a saturated calomel electrode and are expressed against the normal hydrogen electrode.   All measurements were made in the presence of air.   The conditions are alike for the various dyes and the supply of air approximates that in the Warburg vessels.   The substrate (glucose + diphosphate) was added to the extract first and this potential measured.   The dyes were then added at the points indicated by arrows.   Other experiments in which the dye was added first and the substrate later gave the same values for the completed system.   The points shown for gallocyanine were obtained in this way because of solubility difficulties.   ○ represents 4.9×10^{-3} M rosinduline.   At the second arrow thionine to a concentration of 4.9×10^{-4} M was added to this same solution; △, 4.9×10^{-3} M 1-naphthol-2-sulfonate indophenol; ●, 4.9×10^{-3} M pyocyanine; ×, 4.9×10^{-3} M brilliant alizarin blue; □, 4.9×10^{-3} M gallocyanine (↓ indicates addition of substrate).

potentials, thus showing again the irrelevance of it.   However, rosinduline is difficultly reduced[2] by the extract so that in spite of

[2] This difficulty is not entirely due to the negative potential, for phenosafranine, which has about the same normal potential, and neutral red, which is more negative, both bring about an increased oxygen consumption.

its very negative normal potential, under aerobic conditions the potential of the extract, as measured at an electrode, is not lowered (Fig. 9); nor is the oxygen consumption increased (Fig. 7). Thus the actual potential is relatively positive. However, if in addition to rosinduline, a dye of a somewhat more positive normal potential, which is more easily reduced (e.g. indigodisulfonate or thionine) is added, the potential is rapidly lowered (Fig. 9) but the inhibition caused by the rosinduline is unchanged. This inhibition is also easily overcome by addition of hexosediphosphate (Fig. 7), and the fermentation proceeds at a rapid rate until CO_2 approximately corresponding to the added diphosphate has been formed. The rate then drops to a very low value. This is true whether the experiment is made aerobically or anaerobically, for this dye is reduced so slowly by the extract that its effect is the same in either case. This, of course, shows that the consumption of oxygen is not necessary for the development of the inhibition.

As in the case of the naphtholsulfonate indophenol this restored fermentation is not just the fermentation of the added diphosphate. The evidence for this is firstly that it is much too rapid and secondly that the fermentation is accompanied by a decrease of the inorganic phosphate in the solution. An example of this decrease is shown in Fig. 7.

Brilliant alizarin blue shows this same type of inhibition. Fig. 8 shows how its effect is reversed by hexosediphosphate. This dye has a quite low normal potential, but it is in part reduced by the extract so it brings about an oxygen consumption (Fig. 8), and markedly lowers the electrode potential (Fig. 9). However, in spite of this low potential, which is certainly not more positive than with gallocyanine, which does not inhibit fermentation, the inhibition is strong.

SUMMARY

The effect of a number of reversibly oxidizable and reducible dyestuffs on the rate of alcoholic fermentation by yeast extract, especially under aerobic conditions, has been studied. The results indicate that the dyes studied may be classified into three groups. The first, consisting of gallocyanine, phenosafranine, and neutral red, brings about an oxygen consumption but does not inhibit fermentation. The second group, pyocyanine, thionine, methylene blue, gallophenine, and indigodisulfonate, differs widely

within itself but is a group to the extent that each dye inhibits aerobic fermentation by bringing about a destruction of enzymes. The third group, 1-naphthol-2-sulfonate indophenol, rosinduline GG, and brilliant alizarin blue, inhibits aerobic fermentation by the ability to suppress the formation of hexosediphosphate. The inhibitory action can be overcome by the addition of hexose-diphosphate. Neither hexosemonophosphate nor adenylpyro-phosphate can replace diphosphate in restoring fermentation after inhibition by these dyes.

No correlation could be found between the inhibiting action of the dyes and either their normal potential, or the actual electrode potential brought about by them in yeast extract. The effect on oxygen consumption depends, with some complications, on the normal potential. Consequently, the oxygen consumption brought about by the various dyes did not correlate with the manner or magnitude of the inhibiting effect on fermentation.

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A METHOD OF STUDYING CONDITIONS WITHIN DIFFUSION LAYERS

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At the present time there is in biology a pressing need for clear definitions of the physical and chemical processes and laws governing the transport of matter across boundaries, such as cell membranes, capillary walls, etc. Ultimately every attempt to study what is hidden behind the terms penetration and permeability has to face the conditions within diffusion layers; *i.e.*, the gradients of concentration and the course of a possible electrical potential gradient in these layers. In order to arrive at any understanding of the biological diffusion processes, it seems necessary first to accumulate knowledge from well defined artificial diffusion systems. It is hoped that the principle and apparatus described in this paper will serve such a purpose.

Previous Methods—The previous methods applied in studies of diffusion are of several different types. For a full survey the reader may be referred to articles by Williams and Cady (1), von Hahn (2), and Jacobs (3). A great many methods have as a basic principle the following: From one initially sharp boundary between the solutions under investigation and the solvent (or a second solution) diffusion was allowed to take place. After a lapse of some time the diffusion boundary had become extended ("blurred") to such a length that it could practically be separated into layers, which were separately subjected to analysis (Fig. 1).

These procedures suffer from several disadvantages.

1. The apparatus must be kept at a very constant temperature and free from vibration to minimize convection.

2. The extent of the diffusion layer varies with time and is never sharply defined.

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3. The concentration values obtained from the analysis of each "slice" are average figures (*cf.* the horizontal dotted lines in Fig. 2, *A*).

4. The procedures do not conveniently allow us to follow the building up of the steady state condition which is approached in many cases of diffusion. After any chemical analysis a new experiment must be set up.

It is true that optical methods could be used to avoid these troubles. They are, however, hardly applicable when dealing with mixtures, as in most cases of interdiffusion.
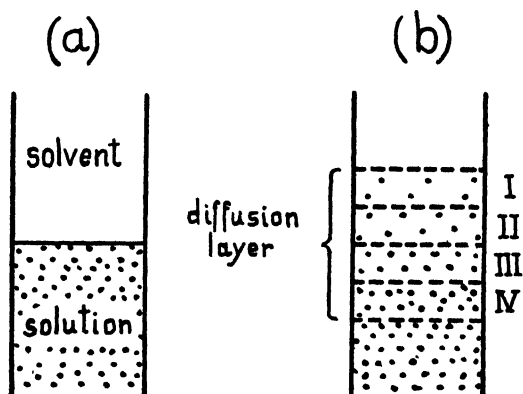
Fig. 1.   A common method of investigating the composition of a diffusion layer: (*a*) initial state with a sharp, free boundary between the two solutions; (*b*) final state where the diffusion layer can be directly analyzed, *e.g.* optically, or chemically after some form of mechanical slicing.   *I*, *II*, *III*, and *IV* indicate "slices."

5. Electrical potential measurements within the diffusion layer are hard to perform without interfering with the progress of the diffusion. Even if it were possible to make the measurements, the figures would have only a qualitative value, because of the circumstances referred to under (2) above.

Particularly points (2) and (3) make an exact quantitative study of a diffusion layer impossible.

The difficulties of (1) and (2) could be avoided by using a porous, rigid diaphragm to separate the (stirred) solutions, a diaphragm which could after conclusion of the experiments be sliced into

layers of definite thickness. A suitable diaphragm for many cases could be obtained by using aqueous gels. Graham in 1862 (4) used a gelatin gel in diffusion columns which he cut up into layers for analysis. Graham's method has later been used in different modifications by several workers (for instance, by Bechhold and Ziegler (5) and in Fricke's (6) refined method for studying Liesegang's structures).

Another type of such a diffusion diaphragm could be simply made by putting several porous membranes close together; for instance, the fritted glass or alundum disks introduced for diffusion studies by Northrop and Anson (7).

The present author has tried the principle of "slicing the membrane," using both gel plugs and porous disks. The results were not satisfactory, because the objections cited above under (3), (4), and (5) were still valid and could not be avoided in practise.

Step Diffusion (Multimembrane) Principle—The new multimembrane method, briefly described previously (8), has been designed to obviate the disadvantages of the older methods, thus permitting a rigorous quantitative study—in the first place of steady state conditions—of diffusion layers in a convenient way. The underlying principle may be made clear by the following qualitative considerations.

Assume that the concentration gradient of one particular substance within a finite, convection-free diffusion layer has attained a steady state; *i.e.*, that no further change occurs with time. This condition may be represented by Fig. 2, *A*. Now the diffusion layer or membrane is cut up into four parallel slices, *I* to *IV*, which are then separated (*cf.* Fig. 2, *B*). If each "chamber," (*1*), (*2*), and (*3*), so formed is immediately filled with a solution of the same composition as that in the plane of the cut ((*1*), (*2*), and (*3*) in Fig. 2, *A*) and if ideal stirring could be maintained in each chamber so as to keep its content perfectly homogeneous (with no unstirred layers),[1] it is obvious that no change of the concentration gradient in the "partial" membranes would take place. On this basis it is easier to explain what will happen if the experiment is *started* with

[1] This cannot be fully realized. The result is that the effective thickness of each slice is increased by an unstirred layer on each side of it. Practically, however, the influence of the unstirred layers can be eliminated (see p. 741).

the multimembrane arrangement instead of with a single membrane. As the concentration gradient plotted in Fig. 2, A as a function of the distance in the diffusion layer represents a state of dynamic equilibrium, exactly the same curve must be approached also when the multimembrane is set up in several sections at the start.
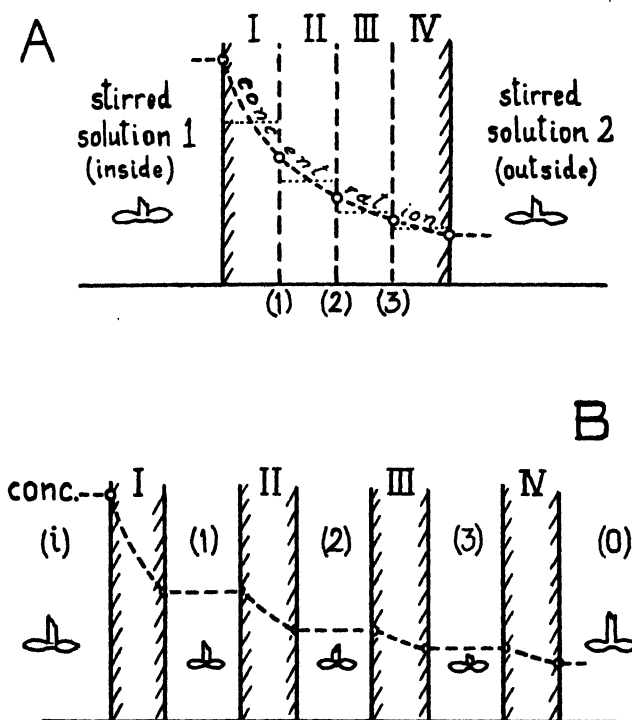
Fig. 2. The step diffusion through a multimembrane: (A) the concentration distribution in a uniform membrane; (B) the same distribution as obtained with the membrane arranged for step diffusion. I, II, III, and IV indicate "slices," and (1), (2), and (3) indicate "chambers." Analyses are performed on the contents of the chambers.

The concentration gradients will be confined to the partial membranes, and it is important to note that the concentration of the content in any "chamber," being the same at the two adjacent membrane surfaces, should correspond to the concentration in one definite *plane* of a single, unsliced membrane (*cf.* Fig. 2). If the

fraction of the total length of the diffusion layer (equal to the sum of the thickness of all partial membranes) occupied by the different partial membranes is known, an exact plot of the concentration-distance function can be made after analysis of the solutions in the chambers. As the volumes of the chambers are immaterial, it is clear that samples for analysis can be withdrawn repeatedly and at any time without disturbing the development of a steady state. Any electrical (diffusion) potential gradients present can be exactly followed simply by inserting suitable measuring electrodes anywhere in the chambers, as these gradients necessarily also are confined to the convection-proof membranes.

This suggested step diffusion across a multimembrane ought evidently to obviate all the disadvantages of previous procedures. The principle put into actual practise has fulfilled the expectations, as will be shown in other papers.

Before starting to describe the apparatus designed, it may be desirable to mention briefly *the kinetics of diffusion in a multimembrane arrangement.*

In order to facilitate the presentation we have so far assumed steady states; *i.e.*, conditions which do not change appreciably with time. The establishment of a steady state requires, strictly speaking, an infinite time, but for practical purposes it may be regarded as attainable within a finite period. In a great many cases the steady state conditions are the interesting ones. If the studies should be performed under conditions far removed from a steady state, the time factor as a variable must be considered. Unfortunately there seems not to have been derived as yet an exact theoretical solution for the *general* problem of diffusion through a single membrane separating two stirred solutions. Explicit expressions for the concentration in the case of a single membrane at any time have been given recently by Barnes (9), valid, however, only for some restricted special cases. The mathematical difficulties encountered appear to be serious even for a single membrane and might be expected to be still worse in any attempt to deal exactly with a multimembrane system.

It would seem, however, that the treatment of the kinetics of step diffusion in a multimembrane would be relatively easy and sufficiently accurate if certain simplifying conditions were introduced, which could be realized practically. The following con-

siderations are limited to non-electrolytes and to cases of electrolyte diffusion in which the influence of electrical forces is negligible.[2]

(a) Thin partial membranes are employed, of equal thickness and porosity.

(b) The "effective" volume, H, of any partial membrane is *small* compared with the constant volume of any chamber, V (in the case treated by Barnes (9) it was desirable to have $H : V < 0.02$).

With these conditions it may be expected that approximately linear concentration gradients will be established within every partial membrane in a short time and will then persist (*cf.* Jacobs (3) p. 74, Barnes (9), Northrop and Anson (7), McBain and Liu (10)). To give a quantitative idea of the significance of the attributes "thin" and "short," we might cite the statement of Jacobs ((3) p. 63) that a substance like NaCl or urea will have a rate of escape from a membrane 0.1 mm. thick, which within 4.56 seconds deviates only 1 per cent from what would be expected in the presence of an exact straight line concentration gradient. The figure for a 1 mm. membrane is 7.6 minutes. With the conditions discussed by Jacobs the corresponding time for a given substance is inversely proportional to its diffusion coefficient, but depends *not* in any way on the absolute concentration.

If the conditions (a) and (b) are fulfilled, the flow of substance across any partial membrane, may, after the lapse of a very short time, be regarded as proportional to the difference of concentration (concentration gradient) between the two solutions surrounding that membrane. This statement is evidently equivalent to Fick's "first law." The chambers in this case have the same rôle as the infinitesimal volume elements in the exact treatment of diffusion kinetics. The concentration in any chamber is dependent upon the rates at which the substance enters and leaves this chamber, which, as just stated, are determined by the concentration gradients. The *rate* of change of concentration can now be said to be proportional to the rate of change of the concentration gradients. This expression might be regarded as of a similar significance as Fick's "second law" or Fourier's general one dimensional diffusion law, in mathematical symbols written

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial x^2}$$

[2] The diffusion is carried out with an over-all salt excess.

(c = concentration, t = time, D = a constant, x = distance in diffusion layer). This is the fundamental diffusion equation.

It might therefore be expected that the step diffusion need not differ formally from diffusion in a homogeneous system, provided the proper assumptions are experimentally realized. Experiments seem to confirm this statement. The parameters employed in any particular solution of the differential equation above are, of course, different in the case of step diffusion.

One experiment with the apparatus described below was set up with the following initial and boundary conditions. A large volume of a stirred HCl solution was placed in contact with a long series of membranes and chambers, all of the chambers having zero concentration at the start. The analyses showed that the propagation of any one particular concentration of the solute along the series of chambers was proportional to the square root of the time.

The "square root of time law" is, as is well known, valid for analogous cases of free diffusion in homogeneous media.

Some Important Factors in Designing the Multimembrane System —There are several requirements to be met.

1. The *diffusion membranes* should be convection-proof and thin in order to obtain rapid results (and if kinetics should be studied, to secure quick establishment of suitable concentration gradients). A lower limit for the thickness is dictated, however, by the demand for freedom from convection and by the presence of so called "unstirred layers." This is bound to exist, due to hydrodynamic reasons, and experiments, to be described in other papers, show that even vigorous stirring does not reduce the "effective" unstirred layer below the order of 0.03 mm., for smooth cellophane membranes. The "unstirred layer effect" has to be reduced to a minimum in exact investigations of certain cases of electrolyte diffusion by selecting a suitable thickness of the membrane (from 0.8 to 0.2 mm.).

2. The membranes should preferably not impose any restraint upon the diffusion or otherwise influence it.

3. The membrane material should be uniform and resistant to the solvent. It ought to be inert also in other respects (for instance, in regard to electrical osmotic effects). The choice of . material may vary widely. Suggestions for porous membranes

are collodion, cellophane, parchment, *Membranfilter,*[3] hardened filter paper, sintered glass, alundum, etc.

4. The volumes of the chambers should be equal and large enough to permit removal of samples for analysis without dimin-
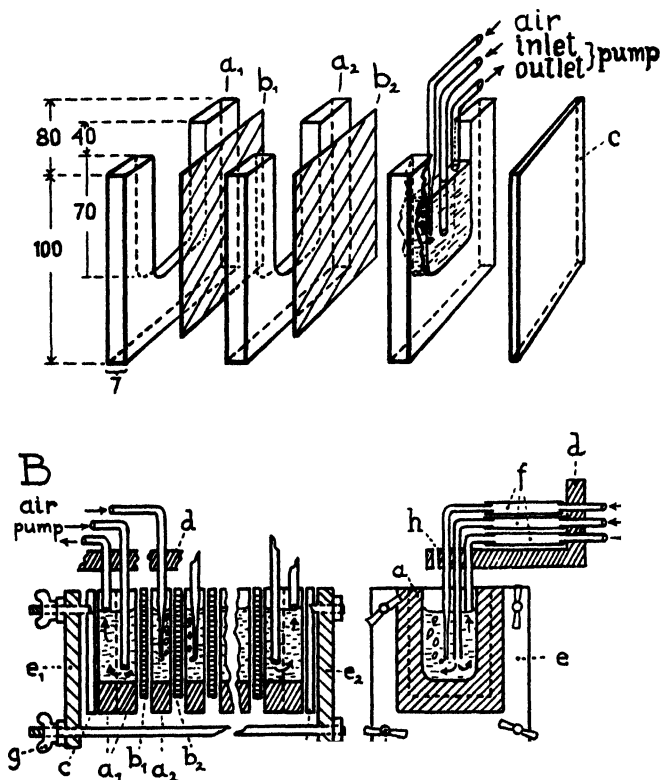
FIG. 3. The step diffusion apparatus: (*A*) showing the principle in the set-up (the dimensions are given in mm.); (*B*) section and side view. *a* rubber washers, *b* porous membranes, *c* glass plates, *d* bakelite rack, *e* bakelite ends, *f* rubber tubings, *g* wing screws, *h* holes for electrodes.

ishing the volume too much. A possible need for a small ratio $H:V$ (*cf.* p. 740) has to be considered.

5. Provision should be made for effective stirring in all solutions and for maintenance of constant composition in any chamber if required.

Apparatus—The requirements described above are satisfactorily met in the design given below. It is obvious that the constructive details can be modified greatly. This apparatus was primarily built for investigations of the steady state conditions in cases of interdiffusion of electrolytes. Evidently it can be employed for a great many other purposes, as determination of diffusion constants,[4] "fractionated diffusion" for preparative purposes, etc.

The body of the apparatus is formed by clamping together a number of membranes, b_1, b_2, etc., between U-shaped washers, a_1, a_2, a_3, etc., as sketched in Fig. 3. Chambers are thus formed which are bounded by two parallel walls of membranes. The end chambers are closed off by glass plates, c. In order to make the chambers tight the U-washers are cut out from semisoft rubber sheets about 7 mm. thick. Furthermore, the membranes are cut about 10 mm. shorter in each square side. When the washers and membranes are pressed together by means of the two U-shaped bakelite ends, e, connected by the wing screws, g, the overlapping parts of the elastic washers are brought into tight contact.

The membranes can, as previously pointed out, be of different material. For substances of low molecular weight, cellophane is very suitable. It seems to have very little influence upon the relative mobilities of the many ions hitherto tried. Still better is the flexible and smooth *Membranfilter*, which can be obtained with porosity from that of the densest cellophane to that of dense filter paper. Hardened filter paper is satisfactory for certain experiments with colloidal substances. Sintered glass plates, alundum, or other porous material may prove useful, particularly for non-aqueous solvents. For quantitative work care should be taken to insure uniformity in porosity and thickness and to avoid disturbance from the "unstirred layers" which even with very vigorous stirring adhere to the membrane surfaces. The influence of the latter factor becomes negligible, however, if the thickness of the membrane exceeds 0.2 mm., as certain experiments indicate. Thicker membranes can be made from thinner simply by super-

[4] The apparatus should be standardized with a substance with known diffusion properties, as is done by, among others, Northrop and Anson (7).

imposition in wet condition, air bubbles and superfluous liquid being expelled.

Every chamber, as well as the outer solutions, has to be stirred thoroughly. Small mechanically driven stirrers may be preferable, but the most convenient method is to bubble air in every chamber through 3 mm. glass tubes drawn out in a capillary, as shown in Fig. 3. The compressed air (which may be obtained from a tap water pump, as described below) is distributed from a
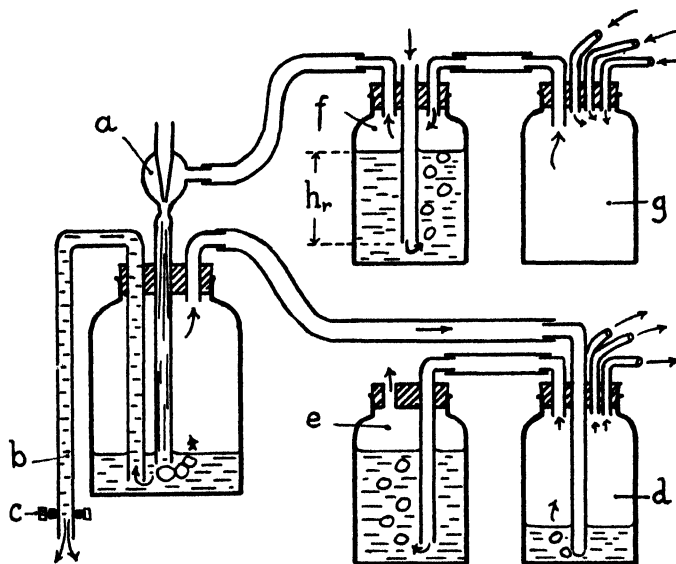
FIG. 4. Arrangement for producing constant air pressure and suction. *a* tap water vacuum pump, *b* water outlet, *c* screw clip for pressure adjustment, *d* air cushion, *e* pressure valve, *f* suction valve, *g* air cushion; h_r should be equal to the height h_p in Fig. 5.

common "air cushion," *d*, as shown in Fig. 4, and the flow regulated by the screw clips.

A continuous replacement of solution in any chamber, as a rule only in the two outside solutions, is accomplished by some form of a circulation pump, feeding and drawing through the tubes as shown in Fig. 3, *A* and *B*. The supply and drain tubes, as well as the air-bubbling tube, are conveniently mounted in holes, *h*, in a bakelite rack, *d*, as sketched in Fig. 3, *B*. Tension by the

elastic rubber tubings, *f*, will cause the glass tubes to remain in any given position in the holes.

The following pump device has proved to be efficient and reliable.

Description of an Air-Driven Circulation Pump without Mobile Parts—This is in principle a combination of the well known air lift
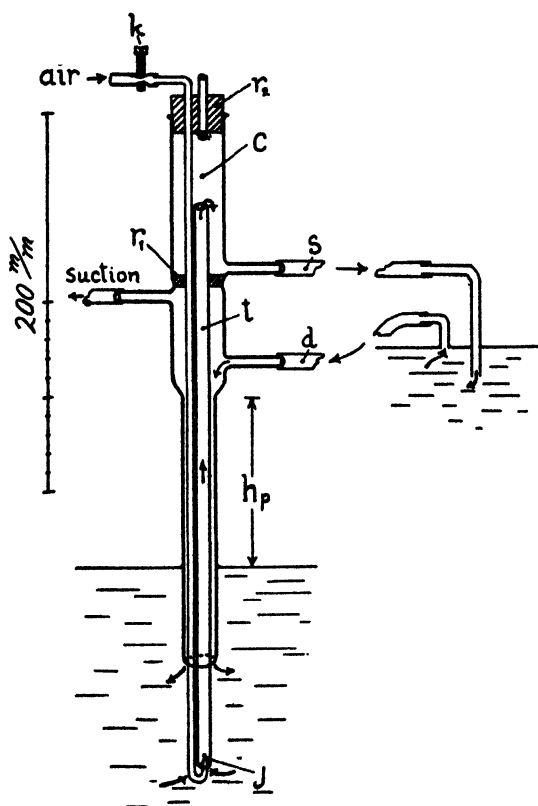
Fig. 5. A simple circulation pump. For explanation, see the text.

pump and a suction device which continuously brings solution from a container to another vessel and simultaneously drains off solution from this vessel back to the container. The details of construction are sketched in Fig. 5. The apparatus is made of glass tubes and rubber stoppers, r_1 and r_2. The compressed air and air suction are supplied from a common vacuum pump at-

tached to a faucet to which a flask has been added in order to obtain air pressure (*cf.* Fig. 4, *a* and *b*). To secure smooth operation with a constant head of suction and pressure, simple pressure regulators and air cushions are provided as shown in Fig. 4. The assembly also provides the air used for the stirring. It is essential to have the head of suction, determined by the depth, h_r (*cf.* Fig. 4) about the same as the height, h_p, in the pump (*cf.* Fig. 5).

The pump operates as follows: The air bubbles formed at the jet, j, push small columns of liquid up through the tube, t, into the top part, c, from which the liquid by gravity flows to its destination by way of the outlet, s. A proper outflow is maintained by adjusting a screw clip on s. The removal of liquid takes place through the tube, d, owing to suction applied to the lower part of the pump. The liquid is there trapped back into the container. There is no danger that the liquid will flow in the wrong direction, provided the suction heads h_p (Fig. 5) and h_r (Fig. 4) are matched and the rate of air bubbling in t is adjusted by the screw clip, k, so as to keep c filled up just to the brim of the tube, t. No appreciable rise of the level above that brim will take place, because any excess liquid will flow back down along the walls of t if proper dimensions are maintained. With the dimensions given in the diagram the pump has a capacity of about 50 cc. per minute; *i.e.*, the ability completely to refill the chamber four to five times per minute.

Operation of Apparatus—The operation in general and the arrangements as to the number and kind of the membranes depend on the nature of the investigation. For the studies of the ionic distribution in the transition layer in interdiffusion of electrolytes four to eight cellophane membranes may be sufficient (each > 0.2 mm. thick; *cf.* p. 741). In other cases a combination of different membranes is suitable; for instance, in certain experiments with colloids the two end membranes may be cellophane or parchment and the intermediate ones may be made of a material permeable to the colloid.

The diffusion vessel is first clamped together and then, from below, placed in position with the stirring and pump device, which is mounted separately on a rack as shown in Fig. 3, *B*.

The stirrer and the pumps are regulated by screw clips on the corresponding rubber tubings. Once properly adjusted for an

experiment, the apparatus can run for days without further adjustment.

A constant composition in regard to one particular substance, if desired, can be maintained in any chamber by means of a continuous drop device leading from a Mariotte's bottle containing a strong solution of that substance.
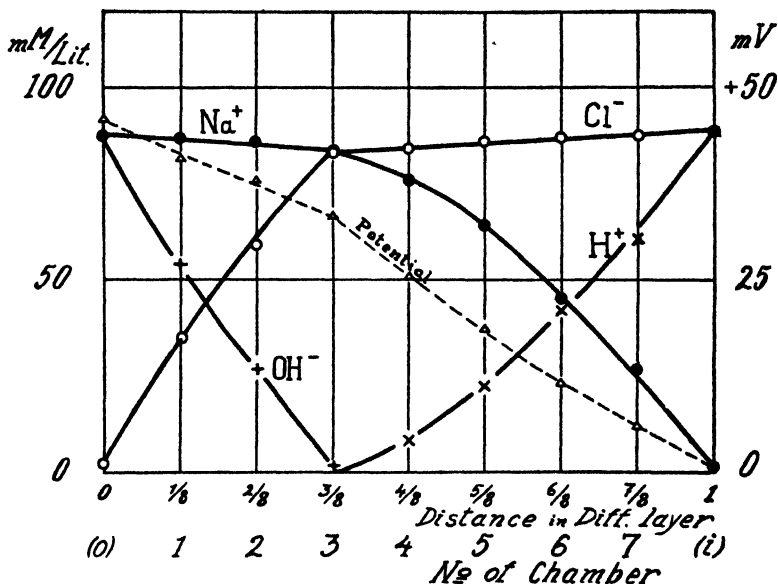
FIG. 6. The diffusion layer in a case of interdiffusion investigated by means of the step diffusion method. Large volumes of HCl and NaOH of equal concentrations were placed outside (*o*) and inside (*i*), respectively, of a multimembrane consisting of eight cellophane sheets. Analyses were made after 24 hours. The vertical lines represent chambers; the spaces between them partial membranes.

. Samples can be withdrawn at any time, and repeatedly. It is preferable to use small amounts (about one-tenth of the content of a chamber) and always withdraw the same volume from each chamber.

Electrical potential gradients can be measured simply by inserting two calomel electrodes, connected to a suitable voltmeter, through the holes, *h*, corresponding to the two chambers between

which the measurement is desired. A convenient and sturdy type of electrode specially designed for this apparatus is described elsewhere (11).

As an example of a result obtained with the apparatus described Fig. 6 is presented, showing the conditions in the diffusion layer in a case of interdiffusion with chemical reactions.

SUMMARY

A principle based upon step diffusion across a multimembrane system has been developed which permits exact quantitative studies of gradients of concentration and electrical potential within diffusion layers.

An apparatus designed according to this principle has been described.

The apparatus may be used particularly for investigations of steady state conditions, but also for kinetic diffusion measurements and for purposes aiming at separation of substances in mixtures.

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COPPER SELENITE AS A CATALYST IN THE KJELDAHL NITROGEN DETERMINATION

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Since the original Kjeldahl technique was developed there have appeared numerous communications relating to modifications designed to speed up and render more complete the conversion of protein nitrogen to ammonia. Selenium alone and in conjunction with conventional catalysts has been used (3, 4, 6, 8). That it is effective in reducing digestion times without apparently altering nitrogen recoveries has been demonstrated (7). Although selenium-copper catalysts are efficacious, the use of the element in conjunction with mercury has been discouraged (1). From the standpoint of manipulative expediency a single compound containing both copper and selenium would be desirable. Such a combination of the two elements is to be found in $CuSeO_3 \cdot 2H_2O$. Copper selenite dihydrate is easily prepared, does not deteriorate or alter upon standing, and is soluble in sulfuric acid. Inasmuch as the Cu:Se ratio represented by the formula had been but casually investigated, it was thought worth while to check the activity of this particular combination in a variety of digestions.

EXPERIMENTAL

Copper selenite dihydrate was prepared by adding copper acetate to a hot solution of selenious acid. The preparation as well as the methods employed in the purification of the starting materials has been previously described (2).

In a series of preliminary analyses on a standard casein sample it was found that when 0.5 gm. samples were digested with 25.0 ml. of H_2SO_4, 12.0 gm. of K_2SO_4, and 0.4 gm. of $CuSeO_3 \cdot 2H_2O$ conversion was complete at the end of 25 minutes. Eight samples

yielded an average of 12.87 ± 0.07 per cent as compared to 12.83 ± 0.03 per cent obtained according to the official method (5).

Although there does not necessarily exist a relationship between clearing time and accuracy of the nitrogen determination (8), the speed of clearing is in most cases a measure of catalytic activity. For purpose of comparison duplicate samples of a homogenized corn-meal were digested under uniform conditions with 25.0 ml. of H_2SO_4, 12 gm. of K_2SO_4, and 0.3 gm. of $CuSeO_3 \cdot 2H_2O$. It was found that with twelve samples weighing between 0.2 and 2.0 gm. the average clearing time was 13 minutes as compared to 23 minutes when the same series was digested with 25.0 ml. of H_2SO_4, 10.0 gm. of K_2SO_4, 0.7 gm. of HgO, and 0.3 gm. of $CuSO_4$(3) and

TABLE I

Sample	Amount used (approximate)	Nitrogen	Nitrogen by other methods (5)
	gm.	*per cent*	*per cent*
Milk powder	1.0	4.96	4.93 (Gunning-Arnold)
Dried blood	0.25	13.45	13.18 (Kjeldahl-Gunning-Arnold)
Brucine	0.5	5.84	6.00 (Calculated)
Wheat flour	1.5	2.00	1.94 (Kjeldahl-Gunning-Arnold)
Bran	1.0	1.94	1.83 (Gunning-Arnold)
Corn-meal	1.0	1.36	1.35 "
Gas vent sludge	1.0	3.69	3.46 (Kjeldahl-Gunning-Arnold)
Sewage sludge	1.2	2.90	2.75 "

17 minutes when 25.0 ml. of H_2SO_4, 10.0 gm. of K_2SO_4, and 0.1 gm. of Se (5) were used.

Some results obtained on a variety of nitrogen-containing products are tabulated in Table I. In the last column are recorded either the calculated percentages of nitrogen or the results obtained upon analysis according to the Gunning-Arnold method or the method of the Association of Official Agricultural Chemists. All results reported are averages of two or more analyses.

SUMMARY

Copper selenite dihydrate is proposed as a single catalyst in the Kjeldahl digestion of nitrogenous materials. It is shown that the time required for clearing and for complete digestion is consider-

ably less in the cases studied than that required when other conventional catalysts are used.

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THE SELECTIVE ADSORPTION OF ENZYMES BY CELLULOSE

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Certain enzymes are more or less readily removed from their solutions by a variety of substances. Pepsin is adsorbed by cholesterol, charcoal, safranine, and fibrin (1). Dyckerhoff and Tewes (2) and Waldschmidt-Leitz and Kofranyi (3) found that crystalline proteins such as edestin or melon globulin are also able to adsorb crystalline pepsin and rennin from their solutions. These authors believe that the crystalline plant proteins remove the active group of pepsin and of rennin from the enzyme-protein molecule. This interpretation, however, has been disproved by Northrop (4) and Sumner (5) in the case of pepsin, and by Tauber and Kleiner (6) in the case of rennin.

Since this paper deals with the adsorption of enzymes, a brief summary concerning the term "adsorption" may be appropriate. Langmuir (7) states: "From the viewpoint adopted in the present paper the forces involved in adsorption, surface tension, etc., are strictly chemical in nature, that is, they do not differ in any essential respect from the forces causing the formation of typical chemical compounds." Many chemists, however, consider adsorption of gases like argon for instance by charcoal (8) or other solid surfaces on a non-chemical basis. In any case Thomas (9) defines adsorption as "the gathering or accumulation of a gas, liquid, or dissolved substance at a surface," disregarding primary valence reactions.

It will be seen from the experiments to be described that pepsin, rennin, and catalase can be removed from their solutions by a single filtration through cotton or to a certain extent by filtration through filter paper, whereas peroxidase is only slightly adsorbed.

The degree of disappearance of enzyme activity and protein, respectively, from the solutions can conveniently be followed by the aid of Kjeldahl technique. The new procedure does not introduce nitrogenous or any other material into the enzyme solution.

<div align="center">EXPERIMENTAL</div>

Adsorption of Crystalline Pepsin on Cotton—20 mg. of dry crystalline pepsin[1] were dissolved in 200 cc. of 0.01 N HCl and adjusted to pH 2.0. Before the adsorption experiment was begun, the solution was twice filtered through a Whatman No. 5, 11 cm. filter paper ("tough and hard, close and very retentive"). Half of it was then filtered once through 3 gm. of dry absorbent cotton (Grade A, Johnson and Johnson) and the first 30 cc. collected.

Activity of Crystalline Pepsin before and after Adsorption—Since the milk-clotting power is a function of the pepsin molecule, the estimation of the milk-coagulating power was employed as a measure of activity. A standard milk of pH 5 may be prepared by adding to cow's milk an equal volume of M acetate buffer of pH 5. This substrate yields comparable results, since it has a uniform pH. 0.3 cc. of the crystalline pepsin solution clotted 10 cc. of milk in 1 minute before filtration through cotton and it took 7 minutes until the same volume of milk was coagulated after one filtration through cotton. This is an adsorption of about 70 per cent. At pH 5, however, there is less adsorption than at pH 2.0. This and all other experiments to follow were carried out at 21°. Nitrogen estimations by the micro-Kjeldahl method before and after adsorption have shown that a definite quantity of extraneous protein was present. This was probably inactive enzyme protein. After adsorption the disappearance of protein nitrogen did not parallel the decrease in activity. The inactive material was perhaps present as a result of the drying process. In the latter acetone is employed. This point, therefore, was not further investigated.

Liberation (Elution) of Pepsin from Cellulose—After adsorption the excess of pepsin solution was removed from the cotton by pressure, and by washing in 6000 cc. of distilled water at 21°.

[1] Kindly furnished by Dr. John H. Northrop.

Water or dilute HCl does not liberate the adsorbed pepsin. The washed cotton possesses peptic activity. The enzyme may be eluted with 0.9 per cent NaCl. Similar results were obtained with a highly active rennin preparation (10).

Adsorption of Commercial Pepsin—This pepsin (Parke, Davis and Company, 1:10,000) is a special preparation. It is highly active and is about one-fourth as potent as the soluble portion of dry crystalline pepsin. It is very soluble in water and dilute HCl. 20 mg. were dissolved in 200 cc. of water and adjusted to pH 2.0 with HCl. The clear solution was twice filtered through Whatman No. 5 filter paper. One-half of it was poured over 3 gm. of cotton and the first 30 cc. were collected. 2.5 cc. of this solution clotted 10 cc. of milk in 4 minutes, whereas before adsorption only 0.3 cc. of the pepsin solution was required to clot the same volume of milk in 4 minutes. This is a removal of about 80 per cent of peptic activity. The micro-Kjeldahl test has shown a decrease of 50 per cent of protein nitrogen after adsorption, indicating that at least 30 per cent of the protein present is not due to pepsin protein.

Adsorption of Catalase—The catalase employed in this experiment was prepared by extracting ground rabbit liver with 5 volumes of 30 per cent alcohol and precipitating the catalase and the proteins with an excess of alcohol containing 20 per cent chloroform. From the precipitate the catalase was extracted with distilled water. The water-clear extract had a pH of 6.9 and the activity was such that 1 cc. decomposed 50 per cent of 10 cc. of a 0.02 N H_2O_2 solution at 2° in 10 minutes in the presence of 2 cc. of 0.05 M phosphate-borate buffer of pH 6.4. Enzyme action was stopped by the addition of 2 cc. of 20 per cent H_2SO_4 and the unchanged H_2O_2 titrated with 0.1 N $KMnO_4$. The filtrate of 50 cc. was completely inactive after filtration through 1 gm. of cotton.

Adsorption of Peroxidase—Peroxidase was prepared by extracting 240 gm. of ground horseradish with 480 cc. of 95 per cent alcohol to which 480 cc. of 0.1 N NaOH had been added. The pH of the filtered extract was 7.8. It was immediately adjusted to 6.3. The precipitate which formed on standing was inactive and therefore discarded. 1 cc. of extract had a potency of 0.1 unit per cc. as determined by the malachite green method (11). Fil-

tration through 1 gm. of cotton removed only a small amount of the peroxidase.

Adsorption by Filtration through Filter Paper—In 1918 Wood (12) noticed that if a piece of filter paper is immersed for some time in a trypsin solution, some of the trypsin is adsorbed. Later Effront (13) showed the same for pepsin. The present author found that there is considerable adsorption by simply filtering dilute solutions of pepsin, rennin, and catalase through filter paper. Peroxidase, however, is only slightly affected. Other enzymes have not been studied yet. A typical experiment is the following: 0.5 cc. of a pepsin solution which had been filtered once through a Whatman No. 5 filter paper clotted 10 cc. of milk in 9 minutes. 1 cc. of the same solution which had been filtered once more through the same filter paper clotted 10 cc. of milk in 8.5 minutes. This is an adsorption of about 50 per cent. All enzyme solutions were water-clear, since they had all first been filtered at least twice through a Whatman No. 5 filter paper.

It is interesting to note that cotton as well as filter paper showed much greater adsorption after moistening with water or, in the case of rennin and pepsin, with dilute HCl. The excess of water or HCl was pressed out, of course.

SUMMARY

Cotton exerts selective adsorption toward enzymes. Its practical application is obvious, since no contamination of the enzyme material occurs, such as takes place with other adsorbents (inorganic gels, tannin, etc.). The use of cotton for filtration of enzyme solutions is therefore impractical, since much of the active enzyme is adsorbed. Cotton should not be used for the filtration of gastric or intestinal juices if enzyme activity is to be determined. If a centrifuge is not available, glass wool or open texture, "fast" filter paper should be used.

Adsorption by cotton may prove useful in the selective concentration of enzymes and in testing the purity of crystalline enzymes.

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THE ERGOT ALKALOIDS*

VIII. THE SYNTHESIS OF 4-CARBOLINE CARBONIC ACIDS

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In a previous preliminary note[1] the results were reported of attempts to synthesize substances closely related to the carboline formula originally suggested by us for lysergic acid. For this purpose we extended the method used by Tatsui[2] and by Akabori and Saito[3] in which tetrahydroharman was produced by the condensation of tryptamine with acetaldehyde. By the substitution of tryptophane itself in this reaction, it has been found possible to prepare in certain cases and with little difficulty 3-substituted tetrahydro-4-carboline-5-carboxylic acids. Thus, tryptophane and formaldehyde yielded *3,4,5,6-tetrahydro-4-carboline-5-carboxylic acid*. Acetaldehyde gave readily isomeric *3-methyl* derivatives due to the formation of a new center of asymmetry at carbon atom (3). The attempt was made to obtain only one of these in a form approaching homogeneity. This substance has also been described by Otani[4] who, however, did not correctly interpret its nature.

With the hope of obtaining a substance approaching the formula assumed for lysergic acid, crotonic aldehyde was then condensed with tryptophane. Although correct analytical figures for a propenyl derivative were obtained with the resulting substance, the latter could not be crystallized and possessed properties which

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* The article (Jacobs, W. A., Craig, L. C., and Rothen, A., *Science*, **83**, 166 (1936)) on ergot alkaloids should have been designated Paper VII of this series.

[1] Jacobs, W. A., and Craig, L. C., *Science*, **82**, 421 (1935).

[2] Tatsui, G., *Chem. Centr.*, **2**, 668 (1928).

[3] Akabori, S., and Saito, K., *Ber. chem. Ges.*, **63**, 2245 (1930).

[4] Otani, S., *Z. physiol. Chem.*, **214**, 30 (1933).

suggested polymerization. By the use of paraldol, the *3-β-hydroxypropyl derivative* was prepared, which crystallized readily. Attempts to dehydrate this substance with formation of a propenyl derivative were unsuccessful. Finally, benzaldehyde gave a crystalline *3-phenyl derivative.*

As a next step in approaching the assumed formula of lysergic acid, which possesses an N-methyl group, methylation of several of these substances was attempted, but the reactions proceeded in undesired directions, giving amorphous products. Better results were obtained with N-methyltryptophane or abrine which was prepared from jequirity beans according to Hoshino.[5] With acetaldehyde, condensation readily occurred but the yield of crystalline   3-methyl-3,4,5,6-tetrahydro-4-methyl-4-carboline-5-carboxylic acid was only about 20 per cent. The major portion apparently remained as a more soluble, difficultly crystallizing stereoisomer which was not isolated as such. Its presence was indicated by oxidation to 4-methylharman as discussed below. Similarly, abrine and benzaldehyde gave a crystalline *3-phenyl derivative.*

Kermack, Perkin, and Robinson[6] have already assumed the intermediate formation of a tetrahydrocarboline derivative in the formation of harman by the condensation of tryptophane with acetaldehyde, but apparently made no attempt to isolate it. They were interested rather in the substance, harman, which was obtained by the additional step of oxidation. Since our tetrahydrocarboline acids were stable, it appeared that decarboxylation must accompany such oxidation. This was found to be the case for, on oxidation of 3-methyltetrahydrocarboline carbonic acid with chromic acid, the base, harman, was directly formed.

Lysergic acid is comparatively stable, requiring a temperature of 200° or more to decarboxylate it. Dihydrolysergic acid, however, shows still less tendency to decarboxylate, since it almost entirely sublimed unchanged at 25 mm. when heated above 300°. If lysergic acid or dihydrolysergic acid were carboline derivatives analogous to the above substances, they should on oxidation

⁵ Hoshino, T., *Ann. Chem.*, **520**, 31 (1935).
⁶ Kermack, W. O., Perkin, W. H., Jr., and Robinson, R., *J. Chem. Soc.*, **119**, 1616 (1921).

(assuming no complication from the previously assumed propenyl group) yield a harman derivative with decarboxylation. This, however, failed to be the case, since the resulting material, although amorphous, was acid in character. With the thought that the N-methyl group might prevent complete oxidation to the harman stage and therefore cause retention of the carboxyl group, a similar oxidation study was made with the above N-methylcarbo-line acid from abrine and acetaldehyde. Decarboxylation, how-ever, occurred and the resulting product proved to be a strong, yellow base, *N-methylharman*, in which the second double bond, as assumed by Nishikawa, Perkin, and Robinson[7] in an analogous case, must lie between carbon atom (3) and a methylene group attached to it. Also, when the mother liquor which contained the majority of the non-crystallizing condensation product of abrine and acetaldehyde was oxidized, a copious yield of the same base was obtained. This indicated the presence of a more soluble stereoisomer due to asymmetry of carbon atom (3).

Finally, contrary to lysergic acid and its dihydro derivative, none of these carboline derivatives gives with dimethylamino-benzaldehyde and hydrochloric acid the reaction generally pro-duced by indole derivatives with α or β positions free. This proved to be the case likewise with yohimbine. As regards the Keller reaction, so characteristic of lysergic acid, only the crotonic aldehyde condensation product gave a color approaching it. The significance of such color reactions, however, is not certain, but the behavior of lysergic acid on oxidation as well as the nature of its degradation products, discussed elsewhere,[8] has caused us to discard the carboline formula for the ergot acid which we had first suggested.

EXPERIMENTAL

3,4,5,6-Tetrahydro-4-Carboline-5-Carboxylic Acid—0.5 gm. of · *l*-tryptophane was treated with 2.5 cc. of N H_2SO_4, 8 cc. of water, and then 2.5 cc. of 40 per cent formaldehyde solution. After a few minutes a copious precipitate developed in the clear solution which first formed. After 1.5 hours at room temperature, a

[7] Nishikawa, N., Perkin, W. H., Jr., and Robinson, R., *J. Chem. Soc.*, **125**, 657 ·(1924).

[8] Jacobs, W. A., and Craig, L. C., *Science*, **83**, 38 (1936).

slight excess of ammonia was added and the mixture was allowed to stand overnight. The precipitate after collection with water was dissolved in 50 per cent alcohol with sufficient ammonia. After diluting somewhat, the alcohol and ammonia were boiled off, when suddenly the crystalline condensation product separated. On repetition of this recrystallization, the substance separated as lustrous leaflets which were anhydrous and melted with effervescence at 310°. Contrary to tryptophane, it gives no color reaction when HCl (1.19) is run down beneath its solution in 1 per cent dimethylaminobenzaldehyde.

$C_{12}H_{12}O_2N_2$. Calculated, C 66.64, H 5.60; found, C 66.94, H 5.37

3,4,5,6-Tetrahydro-3-Methyl-4-Carboline-5-Carboxylic Acid—1 gm. of *l*-tryptophane was warmed with 5 cc. of N H_2SO_4 and 25 cc. of 10 per cent acetaldehyde at 40° for 30 minutes. The clear solution was then heated on the steam bath for 1.5 hours during which crystallization occurred. On evaporation of the solution to remove excess aldehyde, crystallization became copious, especially after addition of sufficient ammonia. The collected material was recrystallized by solution in hot dilute ammonia and boiling off most of the ammonia after clearing with bone-black. The acid separated as cream-colored needles which decomposed at 297° after softening and darkening above 282°. For analysis it was dried at 110° and 15 mm.

$[\alpha]_D^{25} = -115°$ (c = 0.505 in 50 per cent pyridine)
$C_{13}H_{14}O_2N_2$. Calculated, C 67.79, H 6.13; found, C 67.66, H 5.96

No attempt was made to fractionate a possible stereoisomer from the mother liquor.

That decarboxylation accompanied oxidation with formation of harman was shown under the following comparatively gentle conditions.

70 mg. of the above carboline acid were dissolved in 18 cc. of boiling water. 3.5 cc. of 10 per cent $K_2Cr_2O_7$ solution were added. A cloudiness soon appeared. 0.7 cc. of acetic acid was added, which appeared to hasten the reaction, and some gas evolution became apparent. The heating was not longer than 1 minute, when the mixture was cooled and then treated with dilute sodium sulfite to reduce the excess reagent. After making alka-

line with sodium carbonate, the mixture was extracted with ether. The latter on concentration gave crystalline prisms and needles which were collected with ether. After recrystallization from dilute alcohol it melted at 234° (uncorrected).

$C_{12}H_{10}N_2$. Calculated, C 79.08, H 5.53; found, C 78.95, H 5.36

3,4,5,6-Tetrahydro-3-β-Hydroxypropyl-4-Carboline-5-Carboxylic Acid—0.1 gm. of tryptophane was dissolved in a mixture of 0.5 cc. of N H_2SO_4 and 1.5 cc. of water. 0.1 gm. (an excess) of paraldol was added and the mixture was warmed a moment on the bath. A yellow color developed and the reaction was evident from the disappearance of the tryptophane reaction. On addition of dilute sodium acetate solution crystallization occurred. Recrystallization was accomplished from dilute ammonia. The acid formed needles which melted at 261°, depending upon the rate of heating.

The substance gave negative dimethylaminobenzaldehyde and Keller reactions. It contained solvent and was dried for analysis at 100° and 15 mm.

$C_{15}H_{18}O_3N_2$. Calculated, C 65.65, H 6.62; found, C 65.32, H 6.57

3,4,5,6-Tetrahydro-3-Phenyl-4-Carboline-5-Carboxylic Acid — Benzaldehyde was similarly condensed with tryptophane, just sufficient alcohol to cause complete solution being used. The mixture was heated for 15 minutes. The substance was recrystallized from hot dilute ammonia. The phenyl derivative formed needles which melted at 223–226°. It contained solvent and was dried for analysis at 100° and 15 mm.

$C_{18}H_{16}O_2N_2$. Calculated, C 73.94, H 5.52; found, C 73.85, H 5.45

3,4,5,6-Tetrahydro-3-Methyl-4-Methyl-4-Carboline-5-Carboxylic Acid—5 gm. of methyl tryptophane (abrine) were treated with .25 cc. of N H_2SO_4 and 110 cc. of 10 per cent acetaldehyde. On warming at 40° for several hours, a clear solution formed. The mixture was heated an additional 30 minutes on the steam bath and then evaporated on the bath to small volume to remove excess aldehyde. A slight excess of ammonia was then added and the concentration continued. Crystallization slowly occurred, especially after seeding. After the material was collected with water, the yield was 0.8 gm. Attempts to obtain more crystalline ma-

terial from the mother liquor were fruitless, except after oxidation, as given below.

After crystallization by concentration of the dilute ammonia solution, the carboline acid formed colorless needles which contained solvent and effervesced at 248° after preliminary softening and darkening.

For analysis it was dried at 120° and 15 mm.

$C_{14}H_{16}O_2N_2$.   Calculated, C 68.81, H 6.60; found, C 68.79, H 6.79

4-Methyl-4-Carboline   (4-N-Methylharman)—0.1 gm. of the previous carboline derivative was dissolved in 2.5 cc. of water, and to the boiling solution 5 cc. of 10 per cent $K_2Cr_2O_7$ were added. After a moment, a slight change in color appeared with development of a slight cloudiness.   1 cc. of acetic acid was added, which hastened the reaction and induced separation of yellow needles. After 2 minutes the solution was cooled.   The substance which was recrystallized from water proved to be the bichromate of methylharman.   On exposure to light and air it darkened to a gray-green.

$(C_{13}H_{12}N_2)_2 \cdot H_2Cr_2O_7$.   Calculated.   C 51.13, H 4.29
　　　　　　　　　　　　Found.   " 51.69, " 4.32

In another experiment the base was isolated as the acid sulfate as follows.   After oxidation as above, an excess of 10 per cent H_2SO_4 was added to the cooled mixture and all chromate ions were reduced with an excess of SO_2.   After boiling off the latter and cooling, the acid sulfate crystallized after addition of H_2SO_4 to about 10 per cent.   The salt was recrystallized by solution in a small volume of hot water and addition of an equal volume of acetone.   It formed practically colorless, lustrous platelets and needles which melted with decomposition at 273°.

$C_{13}H_{12}N_2 \cdot H_2SO_4$.   Calculated, C 53.03, H 4.80; found, C 53.52, H 4.45

The free base proved to be a strong base, since it was not precipitated from solutions of its salts by ammonia or sodium carbonate.   NaOH, however, caused a change in color to deep yellow with immediate precipitation of the base as yellow needles.

In the preparation of the above crystalline carboline acid from abrine, only 0.8 gm. was obtained from 5 gm. of abrine.   The mother

liquor of the crude crystalline product was diluted to 800 cc. and then after heating to 100° was oxidized with 300 cc. of 10 per cent $K_2Cr_2O_7$ and 40 cc. of acetic acid. After cooling, the excess chromate was reduced with SO_2 and the mixture was strongly acidified with H_2SO_4 to about 10 per cent. The acid sulfate crystallized copiously. After collection it was converted into the free base by precipitation of its aqueous solution with alkali. The yield of the latter was 2.3 gm.

The base was recrystallized from chloroform-petroleum ether and formed yellow needles which melted at 180° after preliminary darkening.

$C_{13}H_{12}N_2$. Calculated, C 79.55, H 6.17; found, C 79.35, H 6.12

3,4,5,6-Tetrahydro-3-Phenyl-4-Methyl-4-Carboline-5-Carboxylic Acid—In the case of benzaldehyde and abrine, 50 per cent alcohol was employed as solvent. Heating on the steam bath for about 18 hours was required before the dimethylaminobenzaldehyde reaction disappeared. The reaction product crystallized on standing. It was dissolved in dilute ammonia and after removal of excess benzaldehyde with ether the solution was acidified with acetic acid. A slight voluminous precipitate was rapidly removed by filtration and the carboline derivative then crystallized as minute needles. It decomposed at 199–201° after preliminary sintering and darkening. It retained solvent tenaciously, and for analysis it was necessary to dry the substance at 140° and 15 mm.

$C_{19}H_{18}O_2N_2$. Calculated, C 74.47, H 5.93; found, C 73.70, H 6.13

THE ERGOT ALKALOIDS

IX. THE STRUCTURE OF LYSERGIC ACID

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The fact that lysergic acid gives a number of reactions usually attributed to certain indole derivatives led us to assume from the start a possible biogenetic relationship of this ergot acid to tryptophane. And, since the alkaloids of the indole group (with the exception of eserine) such as harmine, yohimbine, and evodiamine have been shown to be closely related as 4-carboline derivatives, it was natural to assume as a working hypothesis that lysergic acid is also such a carboline derivative.[1] This view seemed to be supported by some of its properties and by the interpretation of several substances formed on alkali fusion of dihydrolysergic acid. The attempt was then made to verify this conclusion by synthesis. The synthesis of tetrahydrocarboline carbonic acids was readily realized from both tryptophane and methyltryptophane (abrine), as reported elsewhere,[2] but the synthetic substances differed in certain respects from lysergic acid or its dihydro derivative. Contrary to the latter, the carboline acids and other carboline derivatives, such as harman and yohimbine, do not give promptly the characteristic color reaction with dimethylaminobenzaldehyde and hydrochloric acid usually given by α- or β-unsubstituted indoles. This fact and other observations which were accumulating have made it necessary to look less favorably on the carboline structure.

There is no question that the indole ring system is contained in the molecule. The ultra-violet absorption spectra curves of dihydrolysergic acid and dihydrolysergol have been recently shown

[1] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **111**, 455 (1935).

[2] Jacobs, W. A., and Craig, L. C., *Science*, **82**, 421 (1935); *J. Biol. Chem.*, **112**, 759 (1936).

by us[3] to be practically superimposable on those of α-β-dimethyl-indole, of one of the synthetic carboline acids, and of yohimbine, all of which possess the indole nucleus in common.   Also, from among the products of the alkali fusion of dihydrolysergic acid a substance was obtained, the picrate of which gave figures suggesting a formula $C_{11}H_{13}N$ for the substance.   In our previous communication,[1] we believed that this substance could be a methyl-ethylindole.   However, its preparation from dihydrolysergic acid has been recently repeated and has been found to be different from synthetic α-methyl-β-ethylindole, since the picrates gave a definite melting point depression when mixed.   Further, it gave a strong test with dimethylaminobenzaldehyde, indicating, contrary to our first assumption, that either the α or the β position is unsubstituted. β-n-Propylindole has also been excluded by comparison with a substance which we prepared synthetically.   The identity of this substance, which is unquestionably a simple indole derivative, is still a subject of inquiry.   The very small yield which can be obtained makes this difficult.

Further significant information has been recently obtained by a study of the behavior of lysergic acid on catalytic hydrogenation. This reaction has been found to be somewhat involved.   It appears that several reactions occur simultaneously.   On shaking in acetic acid solution with Adams and Shriner's catalyst in an atmosphere of hydrogen, the solution rapidly develops a strong blue-violet fluorescence which persists until the absorption has slowed up and has almost reached the 2 mole stage.   The nature of the material causing this fluorescence has not been determined.   The only crystalline substance which could be isolated was dihydrolysergic acid in a yield of about 50 per cent.   This appeared to be identical in all respects with the dihydrolysergic acid which has already been described as a product of the reduction of lysergic acid with sodium and amyl alcohol.[4]   This identity was confirmed by comparison of the methyl esters from both sources.   When the attempt was made to hydrogenate further, with dihydrolysergic acid itself, absorption was very slow and ultimately the characteristic indole reaction disappeared.   No characteristic product of the reaction could be isolated from the reaction mixture.

[3] Jacobs, W. A., Craig, L. C., and Rothen, A., *Science*, **83**, 166 (1936).

[4] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **106**, 398 (1934).

Similarly, in an experiment where dihydrolysergic acid was reduced with palladium black and hydrogen in acetic acid solution, a second crystalline substance was isolated from the mother liquor of unchanged dihydrolysergic acid in very small yield, which gave practically no dimethylaminobenzaldehyde reaction and was apparently the product of further hydrogenation in the indole nucleus. Thus, no evidence could be obtained for the presence of a double bond other than those contained in the indole nucleus and the easily reduced double bond of lysergic acid. This behavior therefore strongly indicates a tetracyclic structure for the latter.

This conclusion fits well with the interpretation of the nature of two substances which we have already reported as products of the degradation of lysergic acid. The base $C_{11}H_{11}N^1$ previously obtained as a product of the alkali fusion of dihydrolysergic acid has now been definitely identified as 1-methyl-5-aminonaphthalene.[5] Its diazonium salt couples to give azo dyes, and likewise it acts as a coupler. The odor and physical properties as well as the comparison of its benzoyl derivative and picrate with the derivatives of the synthetic base confirmed this identity. The further oxidation of the methyl group to carboxyl with formation of an aminonaphthoic acid was apparently prevented by the rapid distillation of the base out of the melt before such secondary changes could occur. This fact strongly indicates the primary nature of this base. Although it is not excluded that opening of a ring and closing in another way, as has been noted on occasion with complex substances, is not excluded, the fact that this base is a naphthylamine makes this possibility appear remote. We are inclined to accept the formation of this substance as good evidence for the presence of two fused 6-membered rings in lysergic acid, which emerge under the conditions of alkali fusion as a naphthalene derivative. Since methylamine has been almost quantitatively collected from the gases evolved during the production of this substance and since there was no suspicion of contamination of the methylnaphthylamine with an N-methyl derivative, the source of the amino group must obviously be sought in the cyclic indole nitrogen which does not carry the methyl group. The formation of an α-naphthylamine derivative thus restricts the

⁵ Veselý, V., Stursa, F., Olejnicek, H., and Rein, E., *Collect. Czechsl. Chem. Communicat.*, **1**, 506 (1929).

position which can be assigned to the fused pyrrole ring in relationship to the two rings liberated as the naphthalene nucleus.

The second substance, the nature of which we believe we have been able to interpret correctly, is the product of the nitric acid oxidation of ergotinine (lysergic acid) for which the formula $C_{14}H_9O_8N^6$ was derived. Titration of this substance had shown it to be tribasic and it was found to contain an N-methyl group. The remaining 2 oxygen atoms had not been determined. More recently, on distillation with soda-lime, this acid has been found to yield an oil, the properties of which immediately suggested quinoline. This was at once verified by its identification as the picrate. It is therefore very probable that the acid, $C_{14}H_9O_8N$, is an *N-methylquinolinebetaine tricarboxylic acid.* The formation of a quinoline acid from that portion of the lysergic acid molecule carrying the N-methyl group by direct nitric acid oxidation strongly indicates that such a ring system preexists in lysergic acid. Although quinoline derivatives are sometimes formed by rearrangement of substituted indoles, this appears scarcely likely under the conditions of the nitric acid oxidation. This would also be incompatible with the fact that the NCH_3 group is the strongly basic group of the molecule. The ring containing this basic group must therefore be the fourth ring of the molecule.

A tetracyclic ring system, although without precedent, may be constructed to satisfy these more recent observations, as given in Formula I.

In our recent discussion[3] of the ultra-violet absorption spectrum of lysergic acid as compared with that of its hydrogenated derivative, dihydrolysergic acid, it was pointed out that the displacement of the bands and maxima definitely indicates conjugation of this double bond with one of those of the indole ring system. Finally, the recent comparison of the behavior of lysergic acid and dihydrolysergic acid on titration against phenolphthalein has shown that whereas the former consumes almost 1 equivalent of alkali,[7] the latter behaves like a saturated substituted amino acid and cannot be titrated, thus indicating the influence of the double bond in lysergic acid on the carboxyl group or the cyclic NCH_3 group. It appears, therefore, that the latter must be contained in a substi-

[6] Jacobs, W. A., *J. Biol. Chem.*, **97**, 739 (1932).

[7] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **104**, 550 (1934).

tuted hydroquinoline ring system. Although further data will
have to be obtained to fix conclusively the exact position of the
extra double bond of lysergic acid and also that of the carboxyl
group in Ring C or D, we believe that the positions assigned in
Formula I can account for the observations which we have thus far
made.

Thus, such a formula could explain the loss of CO_2 and
methylamine on pyrolysis of lysergic acid (at 210–230°), whereas
the dihydro acid is far more stable. The formation of 1-methyl-5-
aminonaphthalene is also apparent from the cleavage of Ring B,
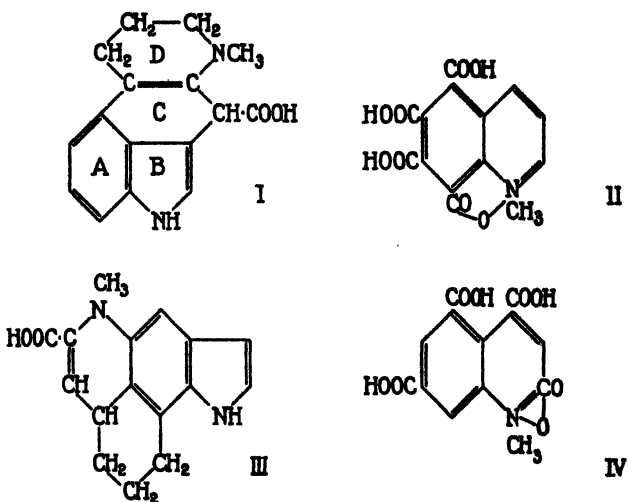
giving the amino group, and Ring D, giving the methyl group.
Finally, the formation of a methylquinolinebetaine tricarbonic
acid, as given in Formula II, by oxidative cleavage of Rings A and
B is a distinct possibility. (Its exact formula, however, is de-
pendent upon the real position of the carboxyl group in lysergic
acid.) And, conversely, the formation of such a quinoline deriva-
tive indicates that the hydroquinoline nucleus as it occurs in
lysergic acid must be substituted in three positions (other than
that occupied by the original carboxyl group of lysergic acid) and
therefore requires such a condensed ring system as given, because
of the restrictions imposed by the empirical formula.

There is an alternative structure, as given in Formula III, which also fits with the assumption of the primary nature of 1-methyl-5-aminonaphthalene and of a quinolinebetaine tricarboxylic acid (Formula IV).   Such a substance could also give an indole derivative, $C_{11}H_{18}N$, unsubstituted in the α and β positions.   However, our experience in the attempt to degrade lysergic acid by exhaustive methylation has given results which appear incompatible with such a structure.   Although the substances encountered were mostly amorphous, their character and behavior were such as to justify conclusions.

β-Dihydrolysergol[8] yields a crystalline *quaternary salt*, $C_{16}H_{20}ON_2 \cdot CH_3I$.   When this salt was decomposed with silver oxide, a base was obtained which on distillation gave an oily distillate which was formed apparently by loss of water and ring cleavage. No crystalline derivative was obtained, but the analysis was in agreement with the required formula of a des-base, *viz.* $C_{17}H_{22}ON_2$. On further methylation the latter gave an addition product (not crystalline) which when treated with silver oxide and then distilled gave trimethylamine.   Unfortunately, the remainder of the molecule was changed to a hopeless resin.   However, the formation of trimethylamine after such a series of steps is in conformity with the exhaustive methylation at that point in the molecule originally consisting of the cyclic NCH_3 group and not involving the indole nitrogen.   Exhaustive methylation with ultimate cleavage of trimethylamine would not be expected from the structure given in Formula III in which the NCH_3 group is directly attached to a benzenoid ring.   Such a substance would behave rather like a tetrahydroquinoline derivative in which rupture between the nitrogen atom and the aromatic nucleus does not usually occur. The observed behavior is consistent with the requirements of Formula I.

A final point which we have attempted to determine is whether lysergic acid occurs conjugated as such in the alkaloid molecule or is formed from a precursor on alkaline hydrolysis.   The formula of the new alkaloid from ergot (ergobasine, ergometrine, etc.) is now established with certainty as $C_{19}H_{23}O_2N_3$.[9]   In agreement

[8] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **108**, 601 (1935).

[9] Stoll, A., and Burckhardt, E., *Compt. rend. Acad.*, **200**, 1680 (1935). Jacobs, W. A., and Craig, L. C., *Science*, **82**, 16 (1935).   Dudley, H. W., *J. Am. Chem. Soc.*, **57**, 2009 (1935).

with this formula is the fact established by us that it is the hydroxyisopropylamide of lysergic acid (or isomer).[9] We have therefore studied the hydrogenation of this alkaloid and its subsequent hydrolysis in order to determine whether the resulting dihydro acid would be identical or different from that obtained on direct hydrogenation of lysergic acid itself. The hydrogenation of the alkaloid proceeded as in the case of lysergic acid and became very slow after 2 moles were absorbed. However, the only crystalline substance which could be isolated was a *dihydro alkaloid*, $C_{19}H_{25}O_2N_3$. This alkaloid on hydrolysis gave a dihydrolysergic acid which proved to be identical in all respects with that obtained from lysergic acid. Although this result does not eliminate the possibility that a shift of the double bond may occur in the transition of these alkaloids to lysergic acid on hydrolysis with alkali, any deep seated rearrangements which involve obscure ring changes appear to be definitely excluded.

<div align="center">EXPERIMENTAL</div>

1-Methyl-5-Aminonaphthalene—400 mg. of dihydrolysergic acid were fused with 2 gm. of potassium hydroxide as previously described,[1] and the heating was continued at 300° for 40 minutes. During this time a small amount of red-colored oil slowly distilled over into the condenser. The condenser was cut from the fusion chamber and extracted with about 1 cc. of ether. The ether extract was shaken with 1 cc. of 10 per cent hydrochloric acid and washed with fresh acid. A crystalline insoluble hydrochloride appeared in the acid layer.

Without filtering, the acid layer containing the crystals was made alkaline with potassium hydroxide and extracted with ether. The ether layer gave 12 mg. of a partly crystalline residue on evaporation. This was fractionated under 0.3 mm. pressure. 10 mg. of an oil which crystallized almost entirely on the condenser distilled up to an oil bath temperature of 160°. This proved to be somewhat impure methylaminonaphthalene. The yield was 4.7 per cent of the theoretical. For recrystallization, the base was dissolved in petroleum ether, concentrated to small volume, and the mixture was chilled. 2.8 mg. of colorless leaves were collected. It began to melt at 65° and was completely melted at 67°. Upon recrystallization from petroleum ether, the melting point was raised to 71.2–72°. A further recrystallization from petroleum

ether gave finally 0.7 mg. of colorless leaves which melted at 71.5–72.8°.

Synthetic 1-methyl-5-aminonaphthalene was prepared according to the direction of Veselý, Stursa, Olejnicek, and Rein.[5] This material melted at 74–74.5° (Veselý *et al.* reported 77–78°). Our substance had the same odor (almost identical with that of α-naphthylamine) and appeared identical in all respects with the synthetic amine. The mixed melting point was 71.5–74.5°. After diazotization, both bases coupled with β-naphthol to give identical colors and, conversely, gave indistinguishable dyes with diazobenzenesulfonic acid.

From the mother liquors of the last two recrystallizations of the base, 2 mg. of base were recovered. This was treated with 2.3 mg. of picric acid in a few drops of ethyl alcohol. Upon cooling, 3 mg. of a yellow picrate were obtained, which began to darken at 200° and melted with decomposition at 208–210°, depending somewhat on the rate of heating. The synthetic picrate was identical in crystalline form and decomposed at 210°. A mixture of the two substances showed no depression. The analysis of this picrate from a previous fusion has been reported.[1] We give these figures again as follows:

$C_{17}H_{14}O_7N_4$. Calculated. C 52.84, H 3.65, N 14.50
                    Found. (*a*) " 53.17, " 3.49, " 14.65
                            (*b*) " 53.30, " 3.55

6 mg. of crude base obtained from another fusion were treated with 4 cc. of 10 per cent sodium hydroxide solution followed by 15 mg. of benzoyl chloride. The mixture was thoroughly shaken and finally warmed until the odor of benzoyl chloride disappeared. The benzoylated product was extracted with ether and the extract was dried with potassium carbonate. The ether solution on concentration to a small volume deposited crystals which were collected with ether. It melted at 163–165°. After recrystallization from ether, the substance melted at 165–167°. A further recrystallization from ethyl alcohol raised the melting point to 168–170°. The mother liquors from the second and third recrystallizations were combined, evaporated to dryness, and recrystallized from ether. This material was used for analysis.

$C_{18}H_{15}ON$. Calculated, C 82.73, H 5.79; found, C 82.62, H 5.54

A benzoyl derivative prepared in exactly the same way from synthetic 1-methyl-5-aminonaphthalene melted at 170–172° (Veselý *et al.* reported 173–174°). The mixture of the substances from both sources melted at 168–170°, and in all other respects the properties of the synthetic substance and that obtained from dihydrolysergic acid were indistinguishable.

Soda-Lime Distillation of the Tribasic Acid, $C_{14}H_9O_3N$—0.15 gm. of the recrystallized acid, $C_{14}H_9O_3N$, obtained by oxidation of ergotinine with nitric acid was ground in a mortar with 0.8 gm. of soda-lime and the mixture was placed in a small apparatus similar to that used in the potassium hydroxide fusion.[1] A current of hydrogen was passed through the apparatus during the reaction. The material was slowly heated with a free flame until decomposition occurred with the distillation of an oil. Evaporation of the hydrochloric acid in the last trap gave no residue which showed the absence of methylamine. The brown-colored distillate in the first trap was washed out with a few drops of ether. The ether extract was dried over potassium carbonate and fractionated. 8 mg. of a colorless oil were collected up to an oil bath temperature of 150° under 25 mm. Most appeared to distil at approximately 140°. It had the odor of quinoline. The yield was thus 13 per cent of the theoretical. The oil was treated with 15 mg. of picric acid, dissolved in ethyl alcohol, and the crystalline picrate was collected with this solvent. 15 mg. of yellow needles were obtained, which melted at 195°. After recrystallization from ethyl alcohol, 9 mg. remained which melted at 197°. A further recrystallization gave a product melting at 198–200°. The crystalline form and properties were indistinguishable from the picrate of synthetic quinoline which melted at 200°. The mixed melting point was at 199–200°.

$C_{15}H_{10}N_4$. Calculated, C 50.28, H 2.81; found, C 50.67, H 2.72

Catalytic Hydrogenation of Lysergic Acid—0.1 gm. of lysergic acid dissolved in 2 cc. of glacial acetic acid was shaken with hydrogen and 25 mg. of Adams and Shriner's catalyst. Absorption of hydrogen occurred rapidly at first but became much slower when 1.5 to 2 moles of hydrogen had been absorbed. At the outset of the reaction a brilliant violet-pink fluorescence developed in the solution but disappeared as the reduction proceeded. The same

color appeared when ergotinine and the alkaloid, as described below, were hydrogenated in this manner. If the reaction was interrupted when 1 mole of hydrogen had been absorbed, the reaction products quickly turned to a deep bluish purple color upon exposure to the air. It was difficult to remove the color from the crystalline material which was isolated. When, however, reduction was allowed to proceed until absorption of hydrogen had become rather slow, the products were quite stable to the atmosphere although the yield of crystalline material was the same as when the reduction was interrupted at an earlier stage. The filtrate from the catalyst was evaporated to dryness under reduced pressure. The residue was heated with 5 cc. of water, and on cooling crystallized. 50 mg. of substance were collected, the properties of which corresponded with those of dihydrolysergic acid previously obtained by us on reduction of lysergic acid with butyl alcohol and sodium.

$[\alpha]_D^{25} = -99°$ ($c = 0.505$ in pyridine)
$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 70.95, H 6.60

The methyl ester prepared from this dihydro derivative with methyl alcoholic hydrochloric acid melted after recrystallization at 181° after slight preliminary softening and gave no depression when mixed with the ester of the acid obtained by the older method.

Catalytic Hydrogenation of the Ergot Alkaloid, $C_{19}H_{23}O_2N_3$— For hydrogenation the alkaloid was carefully recrystallized by addition of chloroform to the concentrated methyl alcoholic solution. 0.1 gm. of this material, which contains 1 mole of chloroform, was placed in the reduction chamber and evaporated two successive times at low pressure with ethyl alcohol in order to remove the chloroform completely. The residue was dissolved in 2 cc. of glacial acetic acid and 50 mg. of Adams and Shriner's catalyst were added. The mixture was shaken under an excess pressure of approximately 1.3 atmospheres of hydrogen. Absorption of hydrogen had become rather slow and the operation was interrupted when 2 moles of hydrogen had been absorbed. The brilliant violet fluorescence which had first developed had become much fainter. The catalyst was filtered off and the filtrate evaporated to dryness. The residue was taken up in dilute sodium

hydroxide and the solution was extracted with hot chloroform. Upon drying the chloroform extract with potassium carbonate and cooling, crystalline needles separated. 50 mg. were collected. The substance sintered considerably at 110° and melted with decomposition at 225–230°, depending somewhat on the rate of heating. The analysis indicated a dihydro derivative. It was dried for analysis *in vacuo* at 140°.

$C_{19}H_{26}O_2N_2$. Calculated, C 69.68, H 7.64; found, C 69.37, H 7.46

Hydrolysis of the Dihydro Alkaloid, $C_{19}H_{26}O_2N_2$—40 mg. of the dihydro derivative were dissolved in 1 cc. of 14 per cent methyl alcoholic potassium hydroxide solution and refluxed for 1 hour. The solvent was evaporated *in vacuo* and the residue was taken up in water. This solution was saturated with carbon dioxide and evaporated again to dryness. The solid residue was extracted with hot ethyl alcohol and the alcoholic extract was then evaporated to dryness. The residue was dissolved in a small volume of water and first made acid to Congo red with sulfuric acid. An excess of ammonium hydroxide was then added and the solution was boiled down to a small volume. Crystals separated on cooling. 15 mg. were collected, which appeared identical in every respect with dihydrolysergic acid.

$[\alpha]_D^{25} = -96°$ ($c = 0.424$ in pyridine)
$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 70.80, H 6.39

Exhaustive Methylation of β-Dihydrolysergol—0.13 gm. of β-dihydrolysergol[8] was dissolved in 5 cc. of methyl alcohol and 2 cc. of methyl iodide were added. After standing 3 hours at 30°, the solvent was evaporated under reduced pressure and the residue was recrystallized from a small volume of methyl alcohol. 0.16 gm. of rhombs or plates was collected. The substance sintered at 250° and melted at 253–254°.

. For analysis it was necessary to dry the substance at 160° and 0.2 mm.

$C_{16}H_{20}ON_2 \cdot CH_3I$. Calculated, C 51.26, H 5.82; found, C 51.53, H 5.53

0.26 gm. of the above methiodide was dissolved in methyl alcohol and treated with silver oxide until the halogen test was negative. Likewise, the filtrate gave no test for silver. The clear, slightly

colored filtrate was evaporated to dryness in a sublimation apparatus. The residue was sublimed under 0.30 mm. The apparatus was attached to a carbon dioxide trap in order to condense any volatile amines. However, none could be detected. Samples for analysis were taken directly from the condenser of the sublimation apparatus.

$C_{17}H_{22}ON_2$.   Calculated.   C 75.56, H 8.21, CH_3 11.11
                Found.   " 75.46, " 7.96, "   10.30

The material on the condenser was removed with methyl alcohol and the solvent was evaporated to dryness. The residue weighed 0.131 gm. and could not be made to crystallize from any solvent. It was dissolved in 3 cc. of methyl alcohol and treated with 1.5 cc. of methyl iodide. After standing 3 hours at 30°, the solution which had become a deep blue was evaporated to dryness under reduced pressure. The residue weighed 0.2 gm. which is approximately the amount calculated for the addition of 1 mole of methyl iodide. The residue was colored and could not be made to crystallize. It was dissolved in methyl alcohol, and the solution was treated with silver oxide as above. Upon evaporation of the filtrate in the sublimation apparatus, a strong odor of trimethylamine became apparent. When an attempt was made to sublime the residue as above under 0.3 mm. pressure, it was converted almost entirely into a non-volatile tar which would not sublime up to 250°. The carbon dioxide trap leading from the sublimation apparatus, however, had a strong odor of trimethylamine and was washed out with dilute hydrochloric acid. The hydrochloric acid was evaporated to dryness, and the residue was dissolved in a minimal volume of 10 per cent hydrochloric acid. The solution was treated with excess gold chloride and 15 mg. of crystalline material were collected with dilute hydrochloric acid. It melted with decomposition at 245°, depending somewhat on the rate of heating.

$(CH_3)_3N \cdot HAuCl_4$.   Calculated.   C 9.02, H 2.51, Au 49.42
                  Found.   " 9.50, " 2.51, " 48.98

A SYNTHESIS OF CONJUGATED BILE ACIDS

II. GLYCODESOXYCHOLIC ACID

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In 1916 Wieland and Sorge (1) first described the formation of addition compounds of desoxycholic acid with fatty acids, and suggested the possible importance of this "choleic acid principle" in furthering the absorption of fatty acids and other substances from the intestinal canal. In human bile, however, desoxycholic acid is united with glycine and taurine; hence it is of considerable importance to determine whether these conjugated acids possess the same combining properties.

In 1902, Wahlgren (2) isolated a substance from ox bile, which he named "glycocholeic acid," because it yielded choleic acid on hydrolysis. At that time, desoxycholic acid and the higher melting choleic acid were thought to be isomeric. As we know from the work of Wieland and Sorge (1) that this choleic acid was a coordination complex of desoxycholic and fatty acids, it is obvious that Wahlgren's product was not pure glycodesoxycholic acid. It will be shown, in this paper, that it was really a mixture of this substance with a small amount of fatty acid, as mixed crystals.

Our work shows there is no difference between natural and synthetic glycodesoxycholic acids. We have made a number of attempts to obtain a coordination compound of glycodesoxycholic and stearic acids, but without success. While desoxycholic acid readily enters into such combination, we found that diformyl-desoxycholic acid and ethyl desoxycholate do not; hence, it is reasonable to infer that both unesterified hydroxyl and carboxyl groups are necessary for the reaction and, therefore, that the conjugated acids will be inactive in this respect. Inasmuch as Wieland (3) has shown that taurodesoxycholic acid also does not

form coordination complexes, we seriously doubt if the "choleic acid principle" really plays an important part in bile itself or in the intestinal digestion of fats.

With regard to the recent article of Sekitoo (4), who claims the isolation of "glycocholeic acid" from rabbit bile, the same criticisms apply as in the case of Wahlgren's work (2). The author must have had mechanically entrained fatty acid (from soaps) in his so called "glycocholeic acid." The "xylene-glycocholeic acid" described was, undoubtedly, impure glycodesoxycholic acid. We also fail to understand why Sekitoo thought the fatty acid he isolated from his supposed "glycocholeic acid" was margaric acid, as he did not exclude the probability of impure stearic acid.

EXPERIMENTAL

Diformyldesoxycholic Acid—This is made according to the procedure described for triformylcholic acid (5). The bath is kept at 60–70°. The residue is crystallized from 300 cc. of boiling 95 per cent alcohol, and 250 cc. of hot water. After a second crystallization and drying at 110° the yield is 45 to 51 gm., or 78 to 89 per cent, m.p. 193–194° corrected (Wieland and Boersch (6), 193°). No solvent of crystallization was present in the air-dried material.

$C_{26}H_{40}O_6$.   Calculated.   C 69.65, H 8.93
448.   Found.[1]   " 69.65, " 8.86

Diformyldesoxycholyl Chloride—The preparation is similar to the one recorded for triformylcholyl chloride (5). These chlorides should not be made when the relative atmospheric humidity exceeds 40 per cent. All operations are best performed in a wide dish placed over fused calcium chloride in a vacuum desiccator. A water pump giving a vacuum of at least 15 mm. is convenient for the evaporations. The gummy residue is rubbed *vigorously* for only 30 seconds at a time, covered again with dry petroleum ether, evaporated, etc.

Diformyldesoxycholyl chloride melts at about 104° corrected with weak foaming. It does not keep. It crystallizes from ben-

[1] All the carbon, hydrogen, and nitrogen analyses reported in this paper were made by Dr. Ing. A. Schoeller in Berlin, Germany.

zene and petroleum ether in rosettes of needles, similar to triformyl-
cholyl chloride, but more nodular in form.

0.4057 gm. dissolved in 95 per cent alcohol required 34.87 cc. of
0.1 N NaOH for neutralization against phenolphthalein. Calcu-
lated, 34.78 cc. (The back titration with HCl is difficult owing to
a premature precipitation before the end-point is reached.)

Desoxycholylamide—This compound has been made by Schenck
(7) by heating desoxycholic acid and ammonia in a sealed tube,
and by Borsche and Schwarz (8) from desoxycholyl azide and
ammonia.

To a solution of 6 gm. of NaOH in 6 cc. of water, 50 cc. of con-
centrated ammonia were added, and then another 150 cc. after
the initial violent reaction had subsided. The acid chloride from
4 gm. of diformyldesoxycholic acid was now stirred in the mixture
for 2 hours, though solution did not take place. The mixture was
acidified with HCl, and the product crystallized from dilute alco-
hol. Yield, 3.8 gm. or 98 per cent. It softens at about 162° and
melts clear and colorless at 188–189° corrected.[2] Recrystalliza-
tion from a mixture of alcohol, acetone, and petroleum ether did
not alter the melting point.

$C_{24}H_{41}O_2N \cdot 3H_2O$ (445). Calculated, N 3.15; found, N 3.18

0.2500 gm. dried at 100° over P_2O_5 at 10 mm. pressure for 5
hours lost 0.0292 gm. in weight, or 11.68 per cent. Calculated,
12.13 per cent for $3H_2O$.

Glycodesoxycholic Acid—This is prepared essentially according
to the procedure described for glycocholic acid (5). The amount
of alkali necessary is best ascertained in a preliminary 1 gm. run,
where 33 to 43 cc., or more, are used. After the period of stirring,
the mixture is warmed at 55° until solution occurs. The crude
product is crystallized from 500 cc. of warm 95 per cent alcohol
with the addition of 2000 cc. of water, previously saturated with
160 cc. of ether. The yield of air-dried acid is 38 to 42 gm. or
73 to 80 per cent. Less ether is used in a second crystallization.

$C_{26}H_{43}O_5N \cdot H_2O$. Calculated. C 66.80, H 9.64, N 3.00
467. Found. " 66.60, " 9.68, " 3.03

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[2] Judging from their behavior on melting, we believe that cholyl- and
desoxycholylamides exist in normal and para forms.

0.7253 gm. dried at 100° over P_2O_5 at 15 mm. pressure for 5 hours lost 0.0313 gm. in weight, or 4.32 per cent. Calculated, 3.85 per cent for 1 molecule of water.

0.6589 gm., air-dried, in 95 per cent alcohol required 14.28 cc. of 0.1 N NaOH with phenolphthalein as indicator. Calculated, 14.20 cc. (We did not experience the difficulty in titration that Wieland (3) mentions.)

The conjugated acid, dried to constant weight, had the following specific rotation.

$$[\alpha]_D^{24} = \frac{+2.26° \times 100}{1 \times 4.710} = +48.0° \text{ (in 95 per cent alcohol) (Wieland (3), } + 48.7°)$$

With regard to the recovery of glycine, we would like to correct two omissions in the previous article (5). The evaporation of the concentrated HCl is followed by an extraction with boiling 95 per cent alcohol; and more glycocholic acid may be obtained by partial evaporation of the original filtrate. Also, all evaporations may be made on the steam bath.

Barium Glycodesoxycholate—To a boiling solution of 1 gm. of sodium glycodesoxycholate in 250 cc. of water are added 6.7 cc. of 10 per cent barium chloride, drop by drop, to a *slight, persistent* cloudiness. The side of the beaker is vigorously scratched at the surface of the boiling mixture until crystallization occurs. After standing overnight, the salt is filtered, washed well with water, and air-dried. Yield, 0.8 gm.[3] Barium glycodesoxycholate crystallizes in balls of fine needles, as Wahlgren (2) described. When crystallized from dilute alcohol, several days are required for complete recovery.

$(C_{26}H_{41}O_5N)_2Ba$ (1033.4). Calculated, Ba 13.30; found, Ba 13.58

The sample for analysis was dried at 100° over P_2O_5 at 15 mm. pressure for 5 hours.

0.5036 gm. dried at 138° over P_2O_5 at 15 mm. pressure for 5 hours lost 0.0350 gm. in weight or 6.95 per cent. Calculated, 6.52 per cent for $4H_2O$.

Crystal Forms and Melting Points of Glycodesoxycholic Acid— Glycodesoxycholic acid is usually obtained from dilute alcohol in

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[3] The first crystals were obtained by seeding the cold, faintly cloudy solution with a crystal of the barium salt of stearic acid-choleic acid.

long needles, in sheaves; occasionally it occurs in irregular crystal fragments or plates with broken and indented edges. Although the two forms are interconvertible by seeding, the needle form usually crystallizes from a solution of the irregular crystal form.

The melting point obtained in the usual way ranges from 180–187° corrected but it is difficult to determine just when fusion occurs. The material melts fairly sharply at 186–187° corrected after it has been boiled in water for 4 hours and air-dried. During this treatment, the material undergoes a marked change in physical appearance, from long lustrous needles to a fluffy, somewhat electrostatic mass of short, ill defined, striated prisms. These probably represent the para form, but when recrystallized from dilute alcohol, the usual needle form in sheaves, or the small irregular plates reappear.

The irregular crystal form sintered when immersed in a bath preheated to 107° but did not swell. A second sample sintered and foamed completely when immersed at 125°. However, the melting point obtained in the usual manner was 184° and ill defined.

A sample of the usual needle form sintered at 107° when immersed in a bath of that temperature, but swelled, resolidified, and remelted at 184° in the usual apparatus. However, only four out of ten attempts were successful in presintering specimens *of the same preparation* at 107°. We thereupon abandoned further attempts to determine more accurately the melting point of the form corresponding to glycocholic acid.

We may say, then, with due reservations, that 107° appears to be the melting point of the normal and 186–187° corrected that of the para form of glycodesoxycholic acid.[4]

Impure glycodesoxycholic acid has a tendency to crystallize in rosettes of short prisms, instead of the usual sheaves of long needles, and, if sufficiently impure, will always crystallize in flower-like rosettes with pointed, elliptical petals.[5]

We have also observed that glycodesoxycholic acid is capable of forming mixed crystals with small amounts of stearic acid, and

[4] The melting point given by Tanaka (9) for the normal form of sodium taurocholate is erroneous and much too high, owing to reasons mentioned in our paper on glycocholic acid (5). This will be further explained in a future communication, together with other errors in Tanaka's paper.

[5] We have also observed impure taurine in this form. These are probably cases of symmetrical distortion.

with the choleic acids of stearic and oleic acids. These crystals appear as rosettes and are perfectly homogeneous when examined microscopically. With larger proportions of the choleic acids, the mixed crystals assume the form of elongated, large hexagonal plates in a milky mother liquor.[6] The addition of ether is also not necessary in crystallizing *pure* glycodesoxycholic acid from alcohol and water.

The preceding facts should be considered in appraising claims for the isolation of any supposed "glycocholeic acid" (2, 4).

Wahlgren's Method of Isolating Glycodesoxycholic Acid from Bile —We have reproduced the basic technique of Wahlgren (2), which consists in the precipitation of the conjugated acid as the barium salt and the *rapid* hot water extraction of the precipitate. Wahlgren makes the definite statement that soaps remain behind in this extraction. However, the following experiment shows that glycodesoxycholic acid cannot be separated from stearic acid in this manner, a procedure which is obviously almost hopeless when other bile salts are present.

To a boiling solution of 1 gm. of sodium glycodesoxycholate and 0.08 gm. of sodium stearate in 200 cc. of water, 10 per cent barium chloride was added in great excess. The gummy precipitate was filtered off rapidly while hot and the clear filtrate was seeded with a crystal of barium glycodesoxycholate and scratched. A cloud of fine needles appeared. The gummy residue was extracted with two 100 cc. portions of boiling water. From the clear filtrates further crops of needles were obtained. The combined mother liquors of the crystals were boiled down to 30 cc. to give a further yield of crystals. Total yield, 0.7 gm., air-dried. The product was treated with hot water and sodium carbonate. After evaporation to dryness and drying in a vacuum desiccator, the residue was extracted with several portions of absolute alcohol. Water was added to deep cloudiness, after acidification to Congo red with HCl. Crystallization set in after stirring in a little ether. The mixed crystals were perfectly homogeneous under the microscope and were in the form of sheaves of needles compounded to appear as large rosettes. Yield, well washed and air-dried,

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[6] It is possible to separate the various types of mixed crystals into their components by careful fractionation from various combinations of alcohol, water, and ether. In some cases HCl is useful in this procedure; in others, detrimental.

0.4 gm., m.p. 178–179° corrected, unaltered by recrystallization from dilute alcohol. A light petroleum ether extract of this product was evaporated to dryness. Characteristic greasy leaflets of stearic acid were obtained. Yield, 8 mg., determined by titration with NaOH. The freed glycodesoxycholic acid now melted 2° higher and after recrystallization gave the usual melting point for the pure acid. Digestion of pure glycodesoxycholic acid with light petroleum ether does not yield an acidic extract.

It is obvious, therefore, that Wahlgren (2) had a mixture as mixed crystals, although the supposed "glycocholeic acid" had an apparently constant melting point and appeared homogeneous. Our work shows that his description of the crystallization of the conjugated acid indicates contamination; e.g., rosette formation, milky solutions, use of ether, etc. Even his analyses allow for an admixture to the extent of 6 per cent of fatty acid.

SUMMARY

1. A new and practical synthesis of glycodesoxycholic acid is described, with an overall yield of 57 to 71 per cent.

2. Desoxycholic acid loses the power to form coordination complexes known as choleic acids, when its hydroxyl or carboxyl groups are masked by the introduction of organic radicals. Hence the existence of "glycocholeic acid" is unlikely.

3. Examination in the literature of descriptions of "glycocholeic acid" from bile shows that they are in accord with impure specimens of glycodesoxycholic acid.

4. There is no difference between natural and synthetic glycodesoxycholic acid.

5. Therefore, the so called "choleic acid principle" is of no practical importance either in digestion or in the cholesterol transport in the bile.

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LACTOFLAVIN, A POSSIBLE CONTAMINANT OF VITAMIN-FREE DIETS

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PLATE 1

The determination of vitamins associated with biological materials and foodstuffs is an important phase of nutritional science. Procedures which involve growth rate as the criterion for determining the presence and amounts of certain vitamins presuppose that the basal rations are totally free from the particular factor being investigated. Many investigators have called attention to the importance of the contaminants possibly present in various constituents of basal rations employed in studies of the water-soluble vitamins of the vitamin B complex. Since casein is the basic protein in most of these investigations, its growth-promoting properties have been critically studied with results which indicate that crude casein and even certain "purified" caseins carry a factor or group of factors which promote growth (1–6). Because the purity of the casein is a factor which may invalidate the results from the use of certain basal diets, various extraction methods involving the use of dilute acetic acid, alcohol, and ether have been designed to free the product from contaminating substances.

In view of the comparatively recent work showing the growth-promoting properties of lactoflavin and its association with milk derivatives, it has seemed desirable to record certain observations, which have been made at these Laboratories, concerning the association of this substance with various "pure" caseins and milk derivatives. The fluorescence characteristics of lactoflavin in "black light" (7, 8) has served as a valuable criterion for determining minute amounts of this growth-promoting vitamin; as

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little as 1 part in 20 million may be detected by the fluorometric method previously reported (8).

The presence or absence of lactoflavin in casein was determined by examining the supernatant liquid from 2 per cent aqueous suspensions in the black light emitted by a Fluoray lamp (7, 8). Fig. 1 shows typical illustrations of the color and intensity of fluorescence exhibited by caseins of varying purity and lactoflavin content. The supernatant liquids in Tubes 2 and 3 show a distinct greenish fluorescence, whereas those in Tubes 1 and 5 show more of a blue fluorescence with a greenish cast; the contents of Tube 4

TABLE I

Correlation of Growth-Promoting Properties of Various Caseins with Their Relative Lactoflavin Content As Indicated by Intensity of Fluorescent Color When Examined in Black Light (Fig. 1)

Tube No.	Sample	Fluorescence of 2 per cent aqueous suspension	Average rate of growth when fed as 18 per cent of basal ration
			gm. per wk.
1	Commercial casein, RX, reprecipitated	Bluish green	4–5
2	Crude commercial casein	Green	6–8
3	Casein-Vitamin-Free, GX	"	4–6
4	" LX	Blue	None
5	Crude commercial casein extracted with acetic acid and alcohol	Bluish green	4
6	Distilled water	None	

are free or substantially free from the greenish fluorescence. The growth rate of white rats, resulting from each of these caseins, when comprising 18 per cent of basal rations supplemented with vitamin B_1, and the antidermatitis factor, as derived from rice polish, is shown in Table I. Fig. 2 shows the character and intensity of the fluorescent color of various water-soluble vitamin concentrates known to contain the antineuritic vitamin B_1 and to possess growth-promoting properties in varying degree and which are usually attributed to the "vitamin G" content. The relative growth-promoting properties of these concentrates, which may be attributed to their lactoflavin content, are shown in Table II.

The methods employed in the purification of caseins determine the efficiency with which the lactoflavin is removed. One particular brand of vitamin-free casein (Casein-Vitamin-Free (Labco)),[1] widely used in biological laboratories, is prepared by a six-step process as a means of rendering it free from the water-soluble vitamins. The process starts with the precipitation of the casein from the milk and the precipitated casein is further subjected to a series of treatments involving elution with weak salt solutions at the respective isoelectric points of the casein. Fig. 3 shows the fluorescence in black light of 2 per cent aqueous sus-

TABLE II

Growth-Promoting Properties of Various Biological Materials Correlated with Their Relative Lactoflavin Content As Indicated by Intensity of Fluorescent Color When Examined in Black Light (Fig. 2)

Tube No.	Material	Concentration	Fluorescent color	Growth response equivalent to lactoflavin (9)
		per cent		*μgm. per gm.*
1	Crude milk vitamin concentrate (2)	0.14	Muddy green	330
2	Rice polish concentrate (10)	1.00	Bluish	None
3	Autolyzed yeast concentrate (No. 11290) (11)	0.82	Brilliant yellowish green	260
4	Vitamin B concentrate (12)	0.82	Muddy greenish brown	140
5	Distilled water			

pensions of samples of these caseins taken at each of the six steps in the process. Tube 1 shows a marked greenish fluorescence, Tube 2 shows only a slight greenish fluorescence, and Tubes 3 to 6, inclusive, show no greenish fluorescence whatsoever. It might be concluded that the caseins in Tubes 3 to 6 are free from the water-soluble lactoflavin. However, this conclusion is not justified, as will be noted from the fluorescent color of the tubes in Fig. 4. The tubes illustrated in Fig. 4 contain the salt eluates from the

[1] The Casein-Vitamin-Free (Labco) is distributed by the Casein Company of America, Inc., New York.

samples of casein taken at the various steps in the process and corresponding to the caseins in the tubes bearing the same numbers as in Fig. 3. A comparison of the tubes in Figs. 3 and 4 shows that the salt eluate from the casein at the third step of the process contains substantial amounts of lactoflavin, as indicated by the marked greenish fluorescence of Tube 3, Fig. 4. The eluates from the fourth, fifth, and sixth steps of the process are entirely free from the greenish fluorescent color, as are also the aqueous elutions of the casein suspensions (Tubes 4, 5, and 6, Fig. 3). These observations would seem to indicate quite conclusively that this type of casein is entirely devoid of the growth-promoting lactoflavin. This visual evidence, as revealed by examination in black light, has also been further confirmed by numerous biological data.

The examination in black light of other substances derived from milk, particularly lactalbumin and milk sugar, has shown that lactoflavin tenaciously adheres to various milk constituents. It is relatively difficult to separate this water-soluble vitamin from the substances with which it is associated in milk, and this is possibly also true for other natural products in which it is found. Dry commercial caseins were not entirely free from lactoflavin after an extended extraction period with weak acetic acid and followed by an extended extraction with alcohol (Fig. 1, Tube 5). This is not surprising in view of the fact that lactoflavin is relatively insoluble in alcohol. During the course of preparation of crystalline lactoflavin (8), the pure product, which had been thoroughly dried, was found to dissolve slowly and with difficulty in alcohol of concentrations varying from 85 per cent to absolute alcohol. It has been possible to obtain crystallization of pure lactoflavin from a 0.05 per cent solution in 96 per cent alcohol.

The data presented do not presume to show the manner in which the lactoflavin is associated with natural substances. However, the relative inefficiency of aqueous and alcoholic extractions suggests that the lactoflavin is not merely adsorbed on a substrate, but may be physically or chemically bound. The possibility that lactoflavin might be a prosthetic group to particular substances of high molecular weight has already been discussed in a recent paper (9).

SUMMARY

Lactoflavin is a contaminant of crude or commercial caseins and even of certain "purified vitamin-free caseins."

Extraction of dry commercial caseins with weak acetic acid and alcohol for extended periods fails to eliminate all of the contaminating lactoflavin; its presence in the "purified" product may be readily revealed by examination in "black light" under proper conditions.

A process of preparing vitamin-free casein, involving a six-step elution treatment with weak sodium chloride solution at the isoelectric point of the casein, results in a product entirely free from lactoflavin.

The relative lactoflavin contents of various caseins and water-soluble vitamin concentrates have been correlated with their growth-promoting properties.

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Fig. 1. Lactoflavin in commercial and "purified" caseins, detected by its fluorescence in black light. Tube 1 represents commercial casein, RX, reprecipitated; Tube 2, crude commercial casein; Tube 3, Casein-Vitamin-Free, GX; Tube 4, Casein-Vitamin-Free, LX; Tube 5, commercial casein extracted with acetic acid and alcohol; Tube 6, distilled water.

Fig. 2. Fluorescent color in black light of various water-soluble vitamin concentrates. Tube 1 represents XXX water-soluble milk vitamin concentrate, 0.14 per cent; Tube 2, vitamin B concentrate obtained from aqueous extraction of rice polish, 1.00 per cent; Tube 3, autolyzed yeast concentrate, 0.82 per cent; Tube 4, vitamin B concentrate from rice polish prepared by the Sure method, 0.82 per cent; Tube 5, distilled water.

Fig. 3. Elimination of lactoflavin from casein (Casein-Vitamin-Free, Labco), as revealed by black light. A 2 per cent aqueous suspension, first step, is indicated by Tube 1; second step, Tube 3; third step, Tube 3; fourth step, Tube 4; fifth step, Tube 5; sixth step, Tube 6.

Fig. 4. Elimination of lactoflavin from casein (Casein-Vitamin-Free, Labco) by elution with sodium chloride, as revealed by black light. Tube 1 represents whey after the removal of casein, first step; Tubes 2 to 6 represent sodium chloride eluate, second to sixth steps respectively.

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